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CORRECTIONS.

On page 284, Vol. lxxviii, No. 2, July, 1928, in Table II, under the heading "pH of Human Innocent Neoplasms at 18°," *Inflammatory tissue from groin* and *Lymphoid tissue* are included. These are not neoplasms, and the low pH values obtained may possibly be due to the enormous numbers of leucocytes in lymphoid and inflammatory tissues, which, as has been shown in Warburg's laboratory,[1] have a power of aerobic glycolysis with the production of lactic acid, similar to that possessed by malignant neoplasms. The mean pH value for innocent neoplasms is not seriously affected if these two values are deleted.

On page 285, in Table III, read *Carcinoma of breast* for *Chronic mastitis of breast.*

On page 286, in the heading to Table IV, read *pH Measurements of Maternal and Fetal Tissues of . . . .* for *pH Measurements of Normal and Fetal Somatic Tissues* of. . . .

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[1] Fleischmann, W., and Kubowitz, S., *Biochem. Z.*, **181**, 395 (1927).

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CONTENTS OF VOLUME LXXVIII.

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ON WALDEN INVERSION.

XII. ON THE OXIDATION OF 3-THIOLVALERIC AND OF 4-THIOLVALERIC ACIDS AND ITS SIGNIFICANCE IN CONNECTION WITH WALDEN INVERSION.

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(Received for publication, March 8, 1928.)

In previous investigations of Levene and Mikeska[1] and of Levene, Mori, and Mikeska[2] a series of thiolcarboxylic acids has been compared with the corresponding sulfo acids with respect to their optical behavior. It was found that the undissociated acids on passing into the ionized state showed a change in rotation the direction of which was identical in the thiol and in the sulfo acids. On the basis of these observations the conclusion was formulated that the change of polarity of the substituting group does not alter the direction of rotation which the original acid displayed on passing from the unionized to the ionized state. In its turn, this conclusion furnished a way of recognizing those reactions of substitution in the simple aliphatic acids which are accompanied by a Walden inversion.

In the series of thiol- and sulfocarboxylic acids previously analyzed, only one exception was encountered. Namely, levo-3-thiolbutyric acid on passing to the mono-ion and then to the di-ion showed a change of rotation towards the right, whereas the sulfo acid derived from it on passing from the undissociated state to the mono- and then to the di-ion changed its rotation to the left. The question naturally arose as to the cause of this exceptional behavior.

* Fellow of the International Education Board.

[1] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1926, lxx, 365.

[2] Levene, P. A., Mori, T., and Mikeska, L. A., *J. Biol. Chem.*, 1927, lxxv, 337.

The difference between the structure of the 3-thiolbutyric acid and the other acids which had been analyzed rests principally in the distance of the thiol group from the carboxyl. Whereas all the other acids were substituted in position (2), this one had the reactive group on carbon atom (3).

Hence, it was necessary to investigate the behavior of a larger group of acids substituted on carbon atoms (3) or (4) and possibly still more distantly from the carboxyl.

The present communication contains a report on the valeric acids substituted in positions (3) or (4).

The configurational relationships of 3- and 4-hydroxyvaleric acids to lactic acid have been established by Levene and Haller.[3]

TABLE I.

Molecular Rotations of Substituted Valeric Acids.

	Hydroxy.	Halide.	Thiol.	Sulfo.	Series.
	degrees	*degrees*	*degrees*	*degrees*	
3-Valeric acid.					
Free acid	+11.4	+19.9	+20.80	+14.32	*l*
Mono-salt	+8.0	+17.3	+13.65	+17.84	
Di-salt			+19.90	+16.06	
4-Valeric acid.					
Free acid	+16.5	+7.1	+6.87	−3.53	*l*
Mono-salt	+3.8	+5.4	+2.43	−3.26	
Di-salt			+2.40	−5.33	

In Table I are given the rotations of the undissociated acids and of the anions of the hydroxy, halogen, thiol, and sulfo acids substituted in positions (3) or (4).

From Table I it is seen that levo-3-thiolvaleric acid, on passing from the undissociated acid to the mono-ion $\left(R\begin{matrix}\diagup COO— \\ \diagdown SH\end{matrix}\right)$ shows a change of rotation to the right, and the latter on passing to the di-ion $\left(R\begin{matrix}\diagup COO— \\ \diagdown S—\end{matrix}\right)$ shows a change of rotation to the left. The corresponding sulfo acid is levorotatory and on passing to the

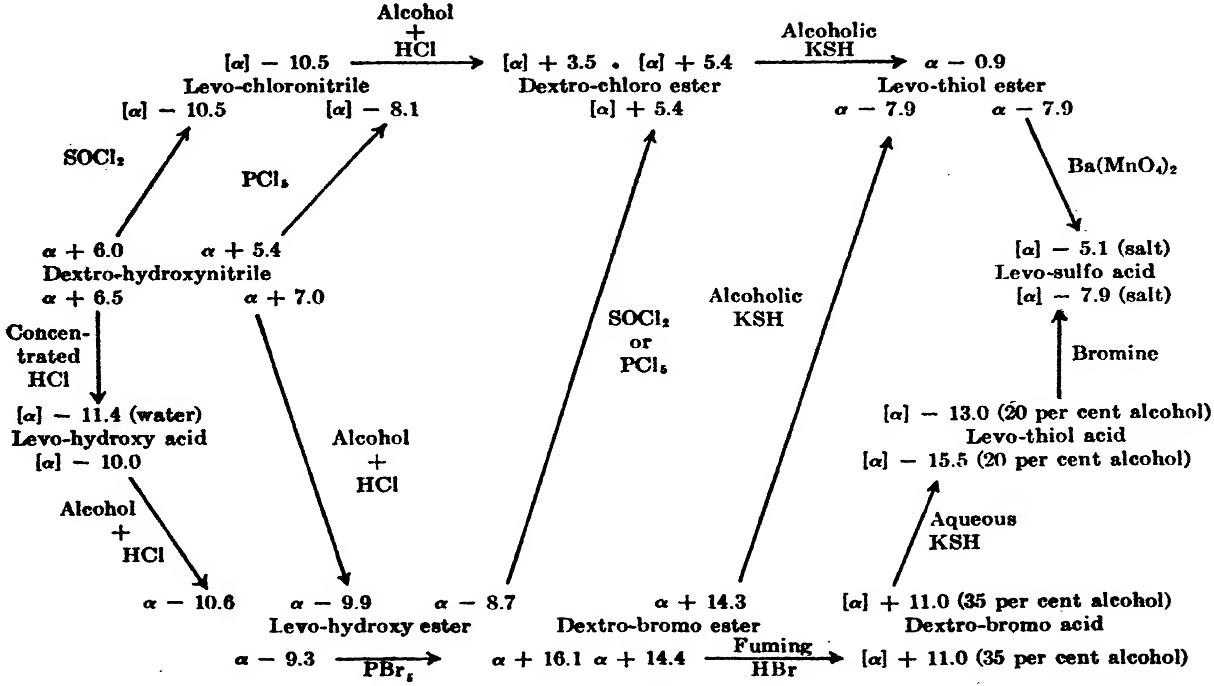
[3] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1926, lxix, 165, 569.

mono-ion $\left(R\begin{matrix}\diagup COOH\\ \diagdown SO_2O-\end{matrix}\right)$ changes its rotation to the left and this on further ionization to the di-ion $\left(R\begin{matrix}\diagup COO-\\ \diagdown SO_2O-\end{matrix}\right)$ changes its rotation to the right. Thus in each case the ionization of the carboxyl leads to a similar change of rotation. This behavior is identical with that of the corresponding acids substituted in position (2).

Levo-4-thiolvaleric acid, on passing to the mono-ion $\left(R\begin{matrix}\diagup COO-\\ \diagdown SH\end{matrix}\right)$ changes its rotation to the right and the latter on further ionization to the di-ion $\left(R\begin{matrix}\diagup COO-\\ \diagdown S-\end{matrix}\right)$ does not change perceptibly. The corresponding sulfo acid is dextrorotatory and on conversion into the mono-ion $\left(R\begin{matrix}\diagup COOH\\ \diagdown SO_2O-\end{matrix}\right)$ changes its rotation to the left, and this on further ionization to the di-ion $\left(R\begin{matrix}\diagup COO-\\ \diagdown SO_2O-\end{matrix}\right)$ changes its rotation to the right. Also in this case the behavior is identical with that of the acids substituted in position (2). Thus, the behavior of the 3-thiol- and 3-sulfobutyric acids remains the only exception.

Thus, on the basis of data on the rotations of thiol- and sulfovaleric acids, it seems to us warranted to say that the halogenation of the hydroxy acids was accompanied by a Walden inversion. Also the substitution of the halogen by the thiol group was accompanied by a Walden inversion. As regards the 3-substituted butyric acid, it seems preferable to postpone judgment until further information is obtained concerning the causes of the exceptional behavior of this substance. Work in this direction is in progress.

It may be mentioned here that difficulties were encountered in the early attempts to substitute the hydroxyl of the free acid by halogen. The difficulty was due to the ready lactonization of 3-substituted acids. To overcome the difficulty the nitrile was



The rotations without solvent are all for 1 dm. tubes. Unless otherwise specified, the solvent used in determining the above rotations was ether. The levo-hydroxy acid used for the preparation of the levo-hydroxy ester was not pure.

halogenated prior to its conversion into the free acid. The chloronitrile was readily converted into the chloro ester and this again into the thiol ester, which then was oxidized to the sulfo ester which in the same process was hydrolyzed to the corresponding acid. There were two unsatisfactory features in this set of reactions. First, the reaction of chlorination was accompanied by a high degree of racemization. Second, the saponification of the thiol ester did not proceed satisfactorily. All difficulties, however, were overcome when the hydroxy ester was brominated and when the bromo acid was converted into the thiol acid. The entire cycle of reactions is given in the accompanying diagram.

Several points brought out on this diagram are worthy of note. First, the fact that the conversion of the hydroxy- or of the chloronitrile into the corresponding acid or ester is accompanied by a change of direction of rotation. This change of rotation is due to the change in polarity of the radicle affected by the reaction. Second, the reaction of halogenation of the nitrile, ester, or acid leads to a halogenated acid of identical configuration. Third, the halogen acid has the identical configuration regardless of the reagent employed for halogenation.

In the experiments with 4-hydroxyvaleric acid, all reactions proceeded smoothly save one; namely, on treatment of ethyl-4-chlorovalerate with potassium hydrogen sulfide, the resulting product was a mixture of the thiol ester with the thiolactone. The latter rotated in the direction opposite to that of the ester. To verify this conclusion pure 4-thiovalerolactone was prepared from 4-thiolvaleric acid.

EXPERIMENTAL.

Part I. β-Substituted n-Valeric Acids.

Levo-β-Hydroxyvaleric Acid.[4]—Chloromethylethyl ketone prepared by the chlorination of methylethyl ketone was converted

[4] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1927, lxxiv, 343. We are indebted to Dr. H. L. Haller for the chloromethylethyl ketone used in these experiments.

into hydroxymethylethyl ketone by the usual method. This compound was reduced to butylene glycol by fermenting bakers' yeast. The best yield of the glycol was 77 gm. from 100 gm. of hydroxymethylethyl ketone. The specific rotations of the glycol were as follows:

$$[\ ]_D^{20} = \frac{+0.87^\circ \times 100}{1 \times 6.00} = +14.5^\circ, \text{ in absolute alcohol.}$$

$$[\alpha]_D^{20} = \frac{+0.08^\circ \times 100}{2 \times 17.3} = +0.23^\circ, \text{ in water.}$$

The glycol ($[\alpha]_D^{20} = +12.4^\circ$ in alcohol) was treated with 1 equivalent of dry hydrogen bromide. From 20 gm. of the glycol 20 gm. of bromohydrin were obtained. The bromohydrin showed the following rotations.

$$\alpha_n^{20} = -10.31^\circ, \text{ without solvent in a 1 dm. tube.}$$

$$[\alpha]_D^{20} = \frac{-0.72^\circ \times 100}{1 \times 10.0} = -7.2^\circ, \text{ in ether.}$$

The bromohydrin ($\alpha_D^{20} = -5.08^\circ$ without solvent in a 1 dm. tube) was refluxed with potassium cyanide in methyl alcohol. The product, β-hydroxyvaleronitrile, showed the following rotations.

$$\alpha_D^{20} = +8.02^\circ, \text{ without solvent in a 1 dm. tube.}$$

$$[\alpha]_D^{20} = \frac{+1.00^\circ \times 100}{1 \times 10.0} = +10.0^\circ, \text{ in ether.}$$

The rotation of the free acid was obtained as follows: A sample of the sodium salt of β-hydroxyvaleric acid obtained by saponification of the nitrile ($\alpha_D^{20} = +6.5^\circ$ without solvent in a 1 dm. tube) was treated with 1 equivalent of hydrochloric acid. This solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.48^\circ \times 100}{2 \times 2.11} = -11.4^\circ.$$

Levo-β-Chlorovaleronitrile.—Since the halogenation of the free hydroxy acid as well as of its salt was not satisfactory, the nitrile was chlorinated.

1. By Means of Thionyl Chloride.—To 5 gm. of dextro-β-hydroxyvaleronitrile ($\alpha_D^{20} = +5.98°$ without solvent in a 1 dm. tube) 7.2 gm. (1.2 mols) of thionyl chloride were slowly added under cooling with an ice-salt mixture. The solution was allowed to stand for 1 hour at room temperature and was then heated on the steam bath with a reflux condenser until sulfur dioxide was no longer evolved (about 15 minutes). The reaction mixture was cooled and poured into about the same quantity of crushed ice and shaken to decompose the unchanged thionyl chloride. The mixture was then extracted with ether. The ethereal extract was washed successively with ice water, with dilute sodium carbonate solution, and with water. After drying with anhydrous sodium sulfate, the ether was removed and the residue was fractionated under reduced pressure (10 mm.). The nitrile boiled at 69–70° and showed the following rotation.

$$[\alpha]_D^{20} = \frac{-1.05° \times 100}{1 \times 10.00} = -10.5°, \text{ in ether.}$$

It is soluble in ether, chloroform, and alcohol, slightly soluble in petrolic ether, and insoluble in water. It analyzed as follows:

0.1194 gm. substance: 0.1528 gm. AgCl.

C_5H_8NCl.	Calculated.	Cl 30.21.
	Found.	" 31.66.

2. By Means of Phosphorus Pentachloride.—10 gm. (1.2 mols) of phosphorus pentachloride were added in small portions to 4 gm. of dextro-β-hydroxyvaleronitrile ($\alpha_D^{20} = +5.35°$ without solvent in a 1 dm. tube) under cooling with an ice-salt mixture. The reaction product was allowed to stand at room temperature until all the pentachloride had disappeared. It was then poured into ice water and the mixture was extracted with ether. The ethereal extract was treated as in the preceding preparation. The nitrile boiled at 68–70° (p = 10 mm.) and gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.55° \times 100}{2 \times 3.4} = -8.1°, \text{ in ether.}$$

The saponification of the chloronitrile and the thiolnitrile (which was prepared by adding alcoholic potassium hydrogen sulfide to the chloro compound) was unsatisfactory, although several methods were tried. Finally the chloronitrile was converted into the ester of the chloro acid as described below.

Levo-Ethyl-β-Hydroxyvalerate.

1. From Levo-β-Hydroxyvaleric Acid.—12 gm. of the levo-hydroxy acid ($[\alpha]_D^{20} = -10°$, in ether), obtained as a syrup by saponification of the hydroxynitrile, were treated with 40 cc. of dry ethyl alcohol containing 1.5 per cent of hydrochloric acid. The solution was gently boiled for 3 hours under a reflux condenser. An aliquot part of the solution was titrated with sodium hydroxide and the calculated amount of sodium ethylate was added to the remainder under cooling. The filtrate from sodium chloride was concentrated under diminished pressure and the residue was taken up with ether. The ethereal solution was dried over sodium sulfate. The residue from the ether was fractionated under reduced pressure. It boiled at 75.5–77° (p = 9.0 mm.). It gave the following rotations.

$$\alpha_D^{20} = -10.63°, \text{ without solvent in a 1 dm. tube.}$$

$$[\alpha]_D^{20} = \frac{-1.56° \times 100}{1 \times 10.00} = -15.6°, \text{ in ether.}$$

The ester is soluble in ether, alcohol, petrolic ether, and chloroform and very slightly soluble in water. It analyzed as follows:

4.155 mg. substance: 8.885 mg. CO_2 and 3.575 mg. H_2O.

$C_7H_{14}O_3$.	Calculated.	C 57.53, H 9.59.
	Found.	" 58.31, " 9.62.

2. From Dextro-β-Hydroxyvaleronitrile.—15 gm. of dextro-hydroxyvaleronitrile ($\alpha_D^{20} = +7.0°$ without solvent in a 1 dm. tube) were dissolved in 75 cc. of absolute (98 to 99 per cent) ethyl alcohol and the solution was saturated with dry hydrogen chloride under cooling with ice. The solution was then heated with a free flame under a return condenser. After 15 minutes ammonium chloride separated. The mixture was boiled 20 minutes longer and then cooled. To the solution some ether was

added and the ammonium chloride was filtered off. The filtrate was evaporated at as low a temperature as possible and the residue was taken up in absolute alcohol. The isolation and purification of the ester were carried out as in the preceding preparation. It boiled at 77–79° (p = 10 mm.). The yield of the crude product was 10 gm. It gave the following rotation.

$$[\alpha]_D^{20} = \frac{-1.47° \times 100}{1 \times 10.00} = -14.7°, \text{ in ether.}$$

It analyzed as follows:

6.205 mg. substance: 13.057 mg. CO_2 and 5.335 mg. H_2O.
$C_7H_{14}O_3$. Calculated. C 57.53, H 9.59.
Found. " 57.38, " 9.62.

Dextro-Ethyl-β-Chlorovalerate.

1. From Levo-β-Chlorovaleronitrile.—4 gm. of the levo-chloronitrile (α_D^{20} = − 8.30° without solvent in a 1 dm. tube) were dissolved in 32 cc. of absolute alcohol saturated with dry hydrogen chloride and the solution was refluxed for 30 minutes. After 5 minutes ammonium chloride separated. The solution was filtered from ammonium chloride and the alcohol was removed by distillation under reduced pressure. The residue was extracted with chloroform. The chloroform extract was dried over sodium sulfate and evaporated under reduced pressure. During the evaporation crystals appeared in the form of plates having a mother-of-pearl luster. Petrolic ether was added and the solution was filtered. The filtrate from the crystals was concentrated and the residue was fractionated under reduced pressure. The ester boiled at 65–67° (p = 10 mm.). The yield was 2.5 gm. It gave the following rotation.

$$[\alpha]_D^{20} = \frac{+0.35° \times 100}{1 \times 10.0} = +3.5°, \text{ in ether.}$$

It is readily soluble in ether, chloroform, petrolic ether, and alcohol, but is insoluble in water. It analyzed as follows:

0.1190 gm. substance: 0.1046 gm. AgCl.
$C_7H_{13}O_2Cl$. Calculated. Cl 21.58.
Found. " 21.74.

The crystals obtained above were purified by dissolving in dry ether and precipitating with petrolic ether. In this state of purity they melted at 100–102° (uncorrected) and gave the following rotation.

$$[\alpha]_D^{20} = \frac{+0.36° \times 100}{2 \times 6.00} = +3.0°, \text{ in chloroform.}$$

The substance analyzed as follows:

0.0988 gm. substance: 0.1028 gm. AgCl.
7.710 mg. " : 0.8377 mg. N (micro Kjeldahl).
$C_5H_{10}OClN$. Calculated. Cl 26.20, N 10.33.
Found. " 25.74, " 10.86.

From the analysis and properties, it seems to us that this substance is β-chlorovaleronamide.

2. From Levo-Ethyl-β-Hydroxyvalerate by Means of Thionyl Chloride.—5 gm. of levo-ethyl-β-hydroxyvalerate ($\alpha_D^{20} = -8.07°$ without solvent in a 1 dm. tube) were treated with 4 gm. (1.2 mols) of thionyl chloride. The isolation and purification were carried out exactly as in the preceding section. It gave the following rotation.

$$\alpha_D^{20} = +6.21°, \text{ without solvent in a 1 dm. tube.}$$

3. From Levo-Ethyl-β-Hydroxyvalerate by Means of Phosphorus Pentachloride.—5 gm. of levo-ethyl-β-hydroxyvalerate ($\alpha_D^{20} = -8.7°$ without solvent in a 1 dm. tube) were dissolved in 10 cc. of dry chloroform. 8.5 gm. (1.2 mols) of phosphorus pentachloride were added under cooling with an ice-salt mixture. The chloro ester was isolated as described above. It boiled at 66.5–67° (p = 10 mm.). It gave the following rotations.

$$\alpha_D^{20} = +6.65°, \text{ without solvent in a 1 dm. tube.}$$

$$[\alpha]_D^{20} = \frac{+0.54° \times 100}{1 \times 10.0} = +5.4°, \text{ in ether.}$$

The substance analyzed as follows:

0.1197 gm. substance: 0.0991 gm. AgCl.
$C_7H_{13}O_2Cl$. Calculated. Cl 21.58.
Found. " 20.48.

Dextro-Ethyl-β-Bromovalerate.—This compound was prepared because the thiol compound from the chloro ester had a low activity. 10 gm. of levo-ethyl-β-hydroxyvalerate ($\alpha_D^{20} = -9.25°$ without solvent in a 1 dm. tube) were dissolved in 10 cc. of chloroform and 35.5 gm. (1.2 mols) of phosphorus pentabromide were then added in small portions under thorough cooling with an ice-salt mixture. The reaction mixture was allowed to stand at 0° with frequent shaking until all the pentabromide had disappeared. The time necessary was usually 3 hours. The bromo ester was isolated as in the preparation of the chloro body. It boiled at 74–76° (p = 10 mm.). The yield was 10 gm. It gave the following rotations.

$$\alpha_D^{20} = +16.1°, \text{ without solvent in a 1 dm. tube.}$$

$$[\alpha]_D^{20} = \frac{+1.08° \times 100}{1 \times 10.00} = +10.8°, \text{ in ether.}$$

The substance analyzed as follows:

0.1052 gm. substance: 0.0964 gm. AgBr.

$C_7H_{13}O_2Br$.	Calculated.	Br 38.28.
	Found.	" 38.99.

It is very soluble in ether, chloroform, petrolic ether, and alcohol but insoluble in water.

Dextro-β-Bromovaleric Acid.—10 gm. of dextro-ethyl-β-bromovalerate ($\alpha_D^{20} = +14.35°$ without solvent in a 1 dm. tube) were treated with 80 cc. of fuming hydrobromic acid under cooling with an ice-salt mixture. The mixture was shaken for 3 days at 10°. It was then poured into about the same quantity of crushed ice and the resulting mixture was extracted several times with chloroform. The chloroform extract was washed with ice water and dried over sodium sulfate. The solvent was evaporated and the residue was fractionated under reduced pressure. After three refractionations the bromo acid boiled at 117–119° (p = 10 mm.). The yield was 3 gm. In another experiment in which the ester was saponified at 40°, the yield was much better, but considerable racemization occurred.

The bromo acid crystallized in glassy plates when allowed to

stand overnight at the temperature of solid carbon dioxide. It melted at about 30°. It is soluble in ether, chloroform, alcohol, and petrolic ether, but insoluble in water. It gave the following rotation.

$$[\alpha]_D^{20} = \frac{+0.66° \times 100}{4 \times 1.50} = +11.0°, [M]_D^{20} = +19.9°, \text{ in 35 per cent alcohol.}$$

To determine the rotation of the sodium salt, 0.2006 gm. of the above bromo acid, which corresponds to 0.225 gm. of sodium salt, was treated with 1 equivalent of sodium hydroxide under cooling and the volume was made up to 15 cc. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{+0.51° \times 100}{4 \times 1.50} = +8.5°, [M]_D^{20} = +17.3°, \text{ in 35 per cent alcohol.}$$

The bromo acid analyzed as follows:

0.1226 gm. substance: 0.1306 gm. AgBr.

$C_5H_9O_2Br$.	Calculated.	Br	44.20.
	Found.	"	45.33.

Levo-Ethyl-β-Thiolvalerate.—7 gm. of dextro-ethyl-β-bromovalerate ($\alpha_D^{20} = +14.25°$ without solvent in a 1 dm. tube) were treated with 33 cc. (3 mols) of alcoholic potassium hydrogen sulfide solution.[5] The mixture was allowed to stand overnight at 0° and then for 2 days at room temperature. It was then heated for 15 minutes on the steam bath to complete the reaction. It was poured into water and the thiol ester was extracted with ether. The ethereal extract was dried over sodium sulfate. After removal of the solvent the residue was fractionated under reduced pressure. It boiled at 75–76°. The yield was 2.5 gm. The residue from the distillation was a thick syrup which was dextrorotatory. The investigation of this substance was not pursued further.

The thiol ester gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.52° \times 100}{1 \times 10.0} = -5.2°, \text{ in ether.}$$

[5] The alcoholic potassium hydrogen sulfide was prepared by dissolving 20 gm. of potassium hydroxide in 100 cc. of absolute alcohol and saturating the solution with hydrogen sulfide under cooling. The total volume was 113 cc.

The thiol ester obtained from the dextro-chloro ester ($[\alpha]_D^{20}$ = + 5.4° in ether) gave a rotation of only α_D^{20} = − 0.90° without solvent in a 1 dm. tube.

2 gm. of the levo-thiol acid ($[\alpha]_D^{20}$ = − 7.75° in absolute alcohol) were dissolved in 12 cc. of absolute alcohol and the solution was saturated with dry hydrogen chloride. After the solution had been allowed to stand for 3 days at 0°, the thiol ester was isolated as usual. It boiled at 71–73° (p = 10 mm.) and gave a rotation of

$$[\alpha]_D^{20} = \frac{-0.99° \times 100}{1 \times 10.00} = -9.9°, \text{ in ether}$$

but it was not analytically pure.[6]

From the above result it seems to us that in the course of esterification some hydrogen sulfide was split off as in the case of the hydrolysis of the thiol ester which will be described later.

The thiol ester gives a strong nitroprusside reaction but no ferric chloride reaction. It is readily soluble in ether, petrolic ether, chloroform, and alcohol, but not in water. The substance analyzed as follows:

0.1155 gm. substance: 0.1619 gm. $BaSO_4$.

$C_7H_{14}O_2S$.	Calculated.	S 19.75.
	Found.	" 19.15.

Levo-β-Thiolvaleric Acid.—The saponification of the thiol ester was not satisfactory either by heating with water or by shaking with concentrated hydrochloric acid in the cold, since it was accompanied by the evolution of hydrogen sulfide. Hence, the thiol acid was prepared from the bromo acid by the usual method.

5.0 gm. of the dextro-bromo acid ($[\alpha]_D^{20}$ = + 7.0° in ether) were treated with 10 cc. (10 mols) of 75 per cent aqueous potassium hydrogen sulfide solution. The mixture was allowed to stand for 1 day at 0° and then heated for 15 minutes on the steam bath. It was extracted with ether (when necessary) and acidified with concentrated hydrochloric acid, whereupon the thiol acid separated as an oil. It was extracted with ether and the combined ethereal extract was washed with water. After drying over

[6] $C_7H_{14}O_2S$. Calculated, S 19.75; found, S 16.63.

sodium sulfate, the solvent was evaporated and the residue was fractionated. The thiol acid boiled at 112–113° (p = 10 mm.). It gave the following rotation.

$$[\alpha]_D^{20} = \frac{-1.24° \times 100}{4 \times 2.00} = -15.5°, [M]_D^{20} = -20.8°, \text{ in 20 per cent alcohol.}$$

For the monosodium salt, 0.2577 gm. of the free acid, which corresponds to 0.3000 gm. of the mono-salt, was treated with 1 equivalent of sodium hydroxide and the volume was made up to 15 cc. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.70° \times 100}{4 \times 2.00} = -8.75°, [M]_D^{20} = -13.65°, \text{ in 20 per cent alcohol.}$$

10 cc. of the above solution were treated with another equivalent of sodium hydroxide and the total volume was made up to 15 cc. This corresponds to 0.2282 gm. of the di-salt. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.68° \times 100}{4 \times 1.52} = -11.2°, [M]_D^{20} = -19.9°, \text{ in 20 per cent alcohol.}$$

The free acid gave both a ferric chloride reaction (deep indigo blue color) and a nitroprusside reaction. It is easily soluble in ether, petrolic ether, chloroform, alcohol, and very slightly soluble in water. The free acid analyzed as follows:

0.1234 gm. substance: 0.2168 gm. $BaSO_4$.
$C_5H_{10}O_2S$. Calculated. S 23.88.
Found. " 24.13.

Levo-β-Sulfovaleric Acid.

1. From Levo-Ethyl-β-Thiolvalerate.—3 gm. of the thiol ester ($\alpha_D^{20} = -7.85°$ without solvent in a 1 dm. tube) were dissolved in a mixture of 30 cc. of acetone and 3 cc. of water. To the solution 6.95 gm. of barium permanganate in 500 cc. of acetone were then added and the mixture was warmed on the steam bath near the end of the reaction. The solution was filtered from manganese dioxide and the latter was washed with acetone and with water alternately. The filtrate and the washings were combined and concentrated under reduced pressure. The residue was taken up

in a little water and extracted with ether to remove a small quantity of oily substance. The aqueous layer was made up to 50 cc. with alcohol (the resultant alcohol was 40 per cent) and this solution was treated with 7 gm. (2 mols calculated on the starting material) of $Ba(OH)_2 \cdot 8H_2O$. The mixture was gently refluxed for 3 hours. It was then diluted with water and neutralized to litmus with dilute sulfuric acid. It was filtered from barium sulfate and the filtrate was evaporated under reduced pressure. The residue was taken up in hot water and alcohol was then added. The barium salt separated first in amorphous and then in crystalline form. After several recrystallizations it crystallized in the form of white prisms and gave the following rotation. 0.4000 gm. of dry barium salt was dissolved in water and the volume was made up to 5 cc.

$$[\alpha]_D^{20} = \frac{-0.81^\circ \times 100}{2 \times 8.00} = -5.06^\circ,\ [M]_D^{20} = -16.06^\circ, \text{ in water.}$$

1 equivalent of hydrochloric acid was added and the solution was diluted to 10 cc. This corresponds to 0.314 gm. of monobarium salt. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.45^\circ \times 100}{2 \times 3.14} = -7.16^\circ,\ [M]_D^{20} = -17.84^\circ, \text{ in water.}$$

To determine the rotation of the free acid, 0.6768 gm. of dry barium salt was treated with 2 equivalents of hydrochloric acid and diluted to 5 cc. with water. This corresponds to 0.4000 gm. of free acid. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{-1.26^\circ \times 100}{2 \times 8.00} = -7.87^\circ,\ [M]_D^{20} = -14.32^\circ, \text{ in water.}$$

The barium salt has no melting point. It is quite soluble in water, but insoluble in alcohol. It analyzed as follows:

0.1000 gm. substance: 0.0106 gm. H_2O.
$C_5H_8O_5SBa \cdot 2H_2O$. Calculated. Water of crystallization 10.18.
Found. " " " 10.60.
0.0894 gm. substance: 0.0654 gm. $BaSO_4$ (for Ba).
0.0887 " " : 0.0642 " " (" S).
$C_5H_8O_5SBa$. Calculated. Ba 43.22, S 10.09.
Found. " 43.05, " 9.94.

2. *From Levo-β-Thiolvaleric Acid.*—1 gm. of the levo-thiol acid was dissolved in 37 cc. (2 equivalents) of 0.4 N barium hydroxide and 9 gm. of barium carbonate (6 equivalents) were then added. The mixture was treated with 4 gm. (a slight excess) of bromine under cooling with ice. The filtrate from the excess of barium carbonate was concentrated to a small volume and alcohol was then added slowly, whereupon the barium sulfonate crystallized out. It was purified by dissolving in a little hot water and precipitating with alcohol. The purified barium salt showed the following rotation.

$$[\alpha]_D^{30} = \frac{-1.20° \times 100}{2 \times 7.58} = -7.92°, \text{ in water.}$$

It analyzed as follows:

0.0943 gm. substance: 0.0688 gm. $BaSO_4$ (for Ba).		
0.1426 " " : 0.0930 " " (" S).		
$C_5H_8O_5SBa$.	Calculated.	Ba 43.22, S 10.09.
	Found.	" 42.93, " 8.96.

Part II. γ-Substituted n-Valeric Acids.

Levo-Ethyl-γ-Chlorovalerate.—Levulinic acid was first prepared and this was reduced to γ-valerolactone according to the directions of Losanitsch.[7] The γ-valerolactone was resolved into its enantiomorphs by means of cinchonidine as described by Levene and Haller.[8] The γ-bromo acid was easily obtained from γ-valerolactone by heating with fuming hydrobromic acid in an autoclave. However, the preparation of the thiol acid from the bromo acid was not successful, although attempted by several methods. The reaction product was always the original substance, *i.e.* γ-valerolactone. Therefore an attempt was made to convert the chloro acid, prepared from its ester as described below, into the thiol acid, inasmuch as the chlorine atom is not so reactive as bromine. Even this reaction was accompanied by the hydrolysis of the chlorine atom. Finally the chloro ester was

[7] Losanitsch, M. S., *Monatsh. Chem.*, 1914, xxxv, 303.

[8] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1926, lxix, 165. We are indebted to Dr. H. L. Haller for the active γ-valerolactone used in these experiments.

prepared essentially according to the directions of Noyes[9] for the corresponding racemic form.

35 gm. of levo-γ-valerolactone ($\alpha_D^{20} = -20.32°$ without solvent in a 1 dm. tube) were dissolved in 140 cc. of absolute ethyl alcohol and the solution was saturated with dry hydrogen chloride under cooling with ice. After standing first at 0° and subsequently at room temperature each for 1 day in a stoppered bottle, the solution was poured on crushed ice, whereupon the ester separated as an oil. It was extracted with petrolic ether and the extract was dried over sodium sulfate. The residue from the ether was fractionated under reduced pressure. It boiled at 71–73° (p = 9 mm.) and gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.79° \times 100}{1 \times 4.00} = -19.7°, \text{ in ether.}$$

The chloro ester obtained from the dextro-lactone ($\alpha_D^{20} = +4.5'$ without solvent in a 1 dm. tube) gave a rotation of $\alpha_D^{20} = +4.33°$ without solvent in a 1 dm. tube and analyzed as follows:

0.1046 gm. substance: 0.0920 gm. AgCl.

$C_7H_{13}O_2Cl$.	Calculated.	Cl 21.58.
	Found.	" 21.76.

Levo-γ-Chlorovaleric Acid.—15 gm. of the levo-chloro ester ($\alpha_D^{20} = -5.37°$ without solvent in a 1 dm. tube) were mixed with 150 cc. of fuming hydrochloric acid and the mixture was shaken at 10° until solution was complete (2 days). The solution was then allowed to stand for 2 days at 40°. After cooling, the same quantity of crushed ice was added and the solution was extracted with chloroform. The chloroform extract was washed once with ice water and dried over sodium sulfate. After three refractionations the chloro acid boiled at 108–111° (p = 10 mm.). It gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.62° \times 100}{2 \times 6.00} = -5.17°, [M]_D^{20} = -7.06°, \text{ in 25 per cent alcohol.}$$

0.2584 gm. of the same sample of the chloro acid was neutralized with 1 equivalent of sodium hydroxide and the volume was made

[9] Noyes, W. A., *J. Am. Chem. Soc.*, 1901, xxiii, 396.

up to 5 cc. under cooling. This corresponds to 0.3000 gm. of sodium salt. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.41^\circ \times 100}{2 \times 6.00} = -3.42^\circ,\ [M]_D^{20} = -5.42^\circ, \text{ in 25 per cent alcohol.}$$

The free acid analyzed as follows:

0.1078 gm. substance: 0.1144 gm. AgCl.

$C_5H_9O_2Cl$.	Calculated.	Cl 26.01
	Found.	" 26.25.

5 gm. of the levo-chloro acid ($\alpha_D^{20} = -6.60^\circ$ without solvent in a 1 dm. tube) were added to 25 cc. of aqueous potassium hydrogen sulfide and the mixture was allowed to stand for 3 days at 0°. An oily substance which separated was extracted with ether and the ethereal extract was dried over sodium sulfate. The residue from the ether was fractionated under reduced pressure. It boiled at 75.5–77° (p = 9 mm.). It contained neither halogen nor sulfur and was found to be γ-valerolactone. It gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.46^\circ \times 100}{1 \times 10.0} = -4.6^\circ, \text{ in ether.}$$

It analyzed as follows:

5.265 mg. substance: 11.650 mg. CO_2 and 3.825 mg. H_2O.

$C_5H_8O_2$.	Calculated.	C 60.00, H 8.00.
	Found.	" 60.34, " 8.12.

Dextro-Ethyl-γ-Thiolvalerate.—30 gm. of the levo-chloro ester ($\alpha_D^{20} = -4.5^\circ$ without solvent in a 1 dm. tube) were treated with 145 cc. (2½ mols) of alcoholic potassium hydrogen sulfide solution. After standing at 0° and at room temperature each for 1 day, the mixture was heated at 150° for 2½ hours in a pressure bottle. The solution was cooled and poured into ice water. The ester was extracted with ether. On removal of the ether, a light yellow mobile oil remained which was fractionated under a pressure of 10 mm. The rotations given below are all for 1 dm. tubes.

F I 73–75°	Weight = 5 gm.	$\alpha_D^{20} = -4.95^\circ$
F II 74.5–76.5°	" = 13 "	$\alpha_D^{20} = -3.96^\circ$
F III 76–80°	" = 5 "	$\alpha_D^{20} = 0^\circ$

F III was redistilled.

F I[I] 78–79° $\alpha_D^{20} = -1.45°$

F II[I] 79–80°

F III[I] 80–83° $\alpha_D^{20} = +2.10°$

F III[I] was again redistilled.

F I[II] 77–80°

F II[II] 81–82° Weight = 1 gm. $[\alpha]_D^{20} = \frac{+0.17° \times 100}{1 \times 10.0} = +1.7°$, in ether.

As above, we obtained dextro- and levorotatory substances which were both neutral to litmus. From the results of analyses and the behavior toward iodine solution the levorotatory substance is the thiolactone and the dextrorotatory is the thiol ester, although the thiolactone was not isolated in a pure state from the above preparation. To make this conclusion certain, the pure thiolactone was prepared from the thiol acid as described later.

The substances analyzed as follows:

F I	0.1146 gm. substance: 0.1913 gm. $BaSO_4$.	Found.	S 23.28.
F I[II]	0.1174 " " : 0.2024 " "	"	" 23.68.
F II[II]	0.1037 " " : 0.1554 " "	"	" 20.59.
	C_5H_8OS (lactone).	Calculated.	S 27.59.
	$C_7H_{14}O_2S$ (ester).	"	" 19.75.

The thiol ester has a quite unpleasant odor and gives a strong nitroprusside reaction but no ferric chloride reaction. It is easily oxidized by iodine; *i.e.*, it decolorizes iodine solution. It is readily soluble in ether, petrolic ether, chloroform, and alcohol, but not in water.

Dextro-γ-Thiolvaleric Acid.—20 gm. of the mixture of the dextrothiol ester and the levo-thiolactone ($\alpha_D^{20} = -19.82°$ without solvent in a 1 dm. tube) were dissolved in 200 cc. of 90 per cent alcohol containing 20 gm. of potassium hydroxide and the solution was heated on the steam bath for 2 hours under a reflux condenser. The excess of alcohol was removed by distillation under reduced pressure. The residue was diluted with ice water and acidified with concentrated hydrochloric acid under cooling, whereupon the thiol acid separated as an oil. It was then extracted with ether and the ethereal extract was dried over sodium sulfate. The thiol acid boiled at 121–122°. These operations should be

performed as quickly as possible after acidifying. It gave the following rotations.

$$[\alpha]_D^{20} = \frac{+0.50° \times 100}{2 \times 4.00} = +6.25°, \text{ in ether.}$$

$$[\alpha]_D^{20} = \frac{+0.42° \times 100}{2 \times 4.09} = +5.14°, [M]_D^{20} = +6.87°, \text{ in 20 per cent alcohol.}$$

For the rotation of the mono-salt, 0.5000 gm. of the same sample was treated with 1 equivalent of sodium hydroxide solution and the volume was made up to 5 cc. This corresponds to 0.582 gm. of the acid salt. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{+0.37° \times 100}{2 \times 11.6} = +1.56°, [M]_D^{20} = +2.43°, \text{ in 20 per cent alcohol.}$$

To the above solution another equivalent of sodium hydroxide was added and the volume was made up to 10 cc. This corresponds to 0.664 gm. of disodium salt. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{+0.18° \times 100}{2 \times 6.64} = +1.36°, [M]_D^{20} = +2.40°, \text{ in 20 per cent alcohol.}$$

The thiol acid is very soluble in ether and alcohol, but very slightly soluble in water. It analyzed as follows:

0.1052 gm. substance: 0.1828 gm. $BaSO_4$.

$C_5H_{10}O_2S$.	Calculated.	S 23.88.
	Found.	" 23.74.

Levo-γ-Thiovalerolactone.[10]—3 gm. of dextro-γ-thiolvaleric acid ($\alpha_D^{20} = +4.87°$ without solvent in a 1 dm. tube) were added to 30 cc. of 10 per cent sulfuric acid and the mixture was shaken for 2 days at 40°. It was then extracted with ether and the ethereal extract was washed with water and dried first with sodium sulfate and subsequently with anhydrous potassium carbonate. After removal of the ether, the thiolactone was fractionated under reduced

[10] The inactive substance was prepared by Fries from valerolactone and phosphorus pentasulfide. Fries, K., and Mengel, H., *Ber. chem. Ges.*, 1912, xlv, 3410.

pressure. It boiled at 69–70° (p = 10 mm.). It gave the following rotation.

$$[\alpha]_D^{20} = \frac{-7.83° \times 100}{1 \times 10.00} = -78.3°, \text{ in ether.}$$

The thiovalerolactone has a not unpleasant odor. It is neutral to litmus, does not decolorize iodine solution, gives no ferric chloride reaction, but gives a nitroprusside reaction. It is soluble in ether, petrolic ether, glacial acetic acid, chloroform, and alcohol, but insoluble in water. The substance analyzed as follows:

0.1015 gm. substance: 0.2144 gm. $BaSO_4$.

C_5H_8OS.	Calculated.	S 27.60.
	Found.	" 29.02.

Levo-γ-Sulfovaleric Acid.—3 gm. of the dextro-γ-thiol acid ($[\alpha]_D^{20}$ = + 6.25°, in ether) were dissolved in 75 cc. (1 equivalent) of 0.3 N barium hydroxide and 26 gm. of barium carbonate (6 equivalents) were added. The mixture was treated with 10 gm. (6 equivalents) of bromine in small portions under cooling. The filtrate from the excess of barium carbonate was concentrated to a small volume under reduced pressure. To the solution alcohol was added, whereupon the barium sulfonate was precipitated as an amorphous substance. The precipitate was purified by dissolving in a little hot water and precipitating with alcohol. This treatment was repeated four times. The barium salt gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.67° \times 100}{2 \times 20.0} = -1.68°,\ [M]_D^{20} = -5.33°.$$

3.0573 gm. of the same sample of barium salt were dissolved in 1 equivalent of hydrochloric acid and the volume was made up to 15 cc. This corresponds to 2.400 gm. of mono-salt. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.84° \times 100}{4 \times 16.0} = -1.31°,\ [M]_D^{20} = -3.26°.$$

For the free sulfo acid, 4.1812 gm. of the same substance were treated with 2 equivalents of hydrochloric acid. This corresponds

to 2.400 gm. of free acid. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{-1.24° \times 100}{4 \times 16.0} = -1.94°, \ [M]_D^{20} = -3.53°.$$

The barium salt is very soluble in water but not in alcohol. It has no melting point. It analyzed as follows:

0.0908 gm. substance: 0.0652 gm. $BaSO_4$ (for Ba).
0.0919 " " : 0.0673 " " (" S).

$C_4H_6O_5SBa$.	Calculated.	Ba 43.22, S 10.09.
	Found.	" 42.25, " 10.06.

STUDIES IN POLYMERIZATION AND CONDENSATION.

III. ON AUTOCONDENSATION OF DIHYDROXYACETONE.

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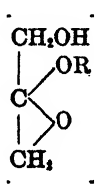
The present work was undertaken because of our interest in the natural tendency of molecules with an ethylene oxidic group to form complexes of high molecular weights and because of our interest in the molecular structures of the substances formed in this manner. Dihydroxyacetone is supposed to crystallize in a dimeric form. To the dimeric half acetals Fischer and his coworkers[1] assign the following ethylene oxidic structure.



Thus dihydroxyacetone is assumed to be capable of combining by molecular forces into polymeric forms. On the other hand, it should be capable of condensing with itself through loss of water into half acetals. The important question then arose as to which of the two reactions predominates when dihydroxyacetone is allowed to stand at ordinary temperature in the absence of other catalytic agents save the oxygen of the air. The immediate occasion for the present study was the following.

Dr. Herzog of H. A. Metz Laboratories, Inc., generously placed at our disposal a quantity of oxanthase which had been kept in their storeroom for about a year and which had turned into a sticky

[1] Fischer, H. O. L., and Mildbrand, H., *Ber. chem. Ges.*, 1924, lvii, 707. Fischer, H. O. L., and Taube, C., *Ber. chem. Ges.*, 1924, lvii, 1502.

mass containing a considerable quantity of crystals. Thus the experiment which we had intended to perform had already taken place and it remained for us to analyze the products formed in the course of this experiment.

Pure dihydroxyacetone was prepared first by Piloty[2] in 1897. From an acetone solution of this substance a crystalline product formed. The observations of Piloty on the conduct of his substance are very significant. The substance formed on the first crystallization and regarded as dihydroxyacetone melted at 68–75°. This indefinite melting point was explained by the assumption of polymerization during the process of melting. The significant point noted in this connection by Piloty was that by repeated recrystallization the melting point of the substance did not become sharper, but on the contrary, became more protracted, the point of complete melting continually rising. Later in his article Piloty describes in greater detail the conduct of dihydroxyacetone on standing. When liquid dihydroxyacetone was allowed to stand without cooling and without seeding with the crystalline material to induce crystallization, it was transformed after a long interval into a crystalline substance which was not dihydroxyacetone but a polymerization or condensation product of it, melting at 155°. When dihydroxyacetone was heated to 60–70°, under reduced pressure, an amorphous substance was obtained which dissolved in water very slowly, but not at all in alcohol. On boiling with dilute mineral acid it dissolved in a cloudy solution which reduced Fehling's solution in the cold. Piloty remarked that the new products were either polymerization or condensation products, but lack of material did not permit him to study the details of their structure.

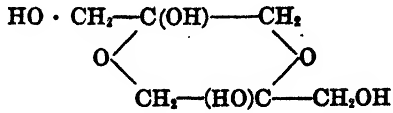
The very important work on dihydroxyacetone which has been done in more recent years was not concerned with the significant findings of Piloty. Bertrand[3] developed a practical method for the preparation of dihydroxyacetone by means of *Bacterium xylinum*. Bertrand made molecular weight estimations of the crystalline dihydroxyacetone by the cryoscopic method and assigned to it a dimeric structure, assuming that the monomeric form was a

[2] Piloty, O., *Ber. chem. Ges.*, 1897, xxx, 3161.

[3] Bertrand, G., *Compt. rend. Acad.*, 1898, cxxvi, 842, 984; *Ann. chim. et physique*, 1904, iii, series 8, 215, 246.

syrup. The crystalline form according to Bertrand melts at about 80° (not sharp).

Wohl and Neuberg[4] suggested for the dimeric form the half acetal structure



Finally Fischer[1] and his coworkers made the important discovery that the substance of Bertrand on distillation at 0.4 to 0.6 mm. pressure at the temperature of the bath at 125–130° yields a syrup which crystallizes in the monomeric form, melting between 65–71°.

From this brief survey it is seen that the very important observations of Piloty have not received the attention which they deserve in the light of the modern speculations regarding the structure of the natural products of high molecular weight.

The material placed at our disposal was a product very similar to the one described by Piloty. Possibly the material had had a chance to undergo further changes than that of Piloty, inasmuch as it had aged for a longer period of time. The sticky material on drying to constant weight at about 75° and reduced pressure contained 42.7 per cent carbon and 5.95 per cent hydrogen. Cryoscopically determined, the molecular weight was about 200, which corresponds to the dimolecular form. It reduced Fehling's solution in the cold. It had no sharp melting point, liquefying at 120°. Its reducing power was about 40 per cent of that of glucose. It gave a triose phenylosazone and a bisulfite derivative. The crude product was fractionated into three parts by means of neutral solvents only, all operations being conducted at room temperature.

Extract I.—Extract obtained from the crude material by extraction with a solution consisting of equal parts of 98.5 per cent alcohol and anhydrous ether.

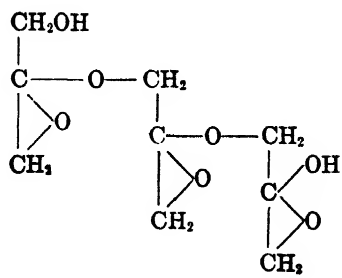
Extract II.—Extract obtained from the first residue by extraction with acetone.

Residue II.—Final residue.

[4] Wohl, A., and Neuberg, C., *Ber. chem. Ges.*, 1900, xxxiii, 3095.

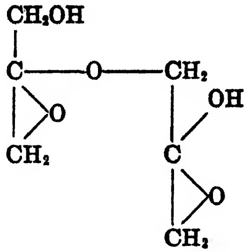
From Extract I were isolated the following substances: volatile fatty acids, the nature of which has not been studied, methylglyoxal, monomeric dihydroxyacetone, and higher condensation products of dihydroxyacetone, the structure of which has not yet been studied in detail. The peculiarity of the monomeric dihydroxyacetone which was obtained by us lay in its melting point which was found quite sharp at 82°. Thus the indefinite melting points reported by previous observers were most likely due to impurities and not to polymerization during the process of melting.

From Extract II also the monomeric form was obtained by distillation at 0.2 to 0.4 mm. pressure and a bath temperature of 120–125°. The residue from the distillation had a sharp melting point at 258°. It was insoluble in water and practically insoluble in all organic solvents, but was soluble in dilute aqueous alkali. It reduced Fehling's solution in the cold; the reducing power in the cold being 11.1 per cent and on warming 22.7 per cent of that of glucose. It did not form an insoluble phenylosazone nor a bisulfite derivative. It had the composition of $C_9H_{14}O_7$, and presumably had the following structure.



Whether this substance was preformed or was developed during the process of distillation is difficult for the present to state with certainty.

From Residue II a crystalline substance was obtained which had a sharp melting point at 164°. It was soluble in cold water, in hot methyl, ethyl, and butyl alcohols and very readily soluble in pyridine. It reduced Fehling's solution in the cold. The substance analyzed exactly for $C_6H_{10}O_5$. This substance then has the following structure.



All reactions leading to this substance were conducted at room temperature, and hence the conclusion is warranted that *on standing, dihydroxyacetone spontaneously condenses through loss of water into substances of higher molecular weight. Polymerization of Bertrand's substance into true polymers of higher molecular weight has not been observed.*

In fact, the present investigation points to the necessity of reinvestigating the nature of Bertrand's substance. Is it actually a dimeric form or is it a mixture of the monomeric form contaminated with some of the condensation products? Indeed, it is not at all clear why the monomeric form isolated by us should have the same melting point as the dimeric form of Bertrand. On the other hand, should the generally accepted view of the molecular state of Bertrand's substance be substantiated, and thus should it be proved that the parent substance of the condensation products is the dimeric dihydroxyacetone, then *the conclusion will be warranted that polymerization is the initial phase in the process of condensation. The true polymers, then, are groupings analogous to Kekulé's giant molecules.*

EXPERIMENTAL.

A. Properties of the Crude Material.

The crude material consisted of crystals imbedded in a thick syrup, the material as a whole having the character of a sticky mass. It was soluble in hot alcohol and in water. The aqueous solution reacted acid to litmus. It reduced Fehling's solution in the cold. The reducing power by the method of Maquenne and Lehmann was 40 per cent of that of glucose. With phenylhydrazine it gave an osazone in the cold.

The composition of the crude mass dried to constant weight under reduced pressure at the temperature of chloroform vapor was as follows:

4.905 mg. substance: 7.680 mg. CO_2 and 2.620 mg. H_2O.
Found. C 42.69, H 5.95.

The molecular weight determined cryoscopically in water was the following.

13.0000 gm. H_2O, 0.2948 gm. substance; 0.210° freezing point depression.
Found, molecular weight 201.
13.0000 gm. H_2O, 0.5540 gm. substance; 0.410° freezing point depression.
Found, molecular weight 197.

B. Fractionation of the Crude Material.

1. Extract I.—The original sticky mass (about 500 gm.) was suspended in 2 liters of a solution consisting of equal parts by volume of alcohol (98.5 per cent) and of anhydrous ether. The supernatant liquid was decanted and the operation was repeated until the remaining residue acquired the character of a slightly soft solid. About ten extractions were required.

(*a*) *Properties of Extract I.*—All extracts were filtered and concentrated under reduced pressure to a thick syrup. This syrup was soluble in water, and in alcohol. An aqueous solution reacted acid to litmus. The aqueous solution formed a phenylosazone in the cold. It formed a bisulfite derivative. The reducing power in the cold of the syrup was 25 per cent of that of glucose.

(*b*) *Fractionation of Extract I.*—The distillation was carried out in the following way. 40.0 gm. of the syrup, which still contained about 20 per cent of alcohol, were introduced into a Claisen flask which had the side tube bent downward almost vertically in a manner to permit convenient insulation of the distilling system by means of asbestos paper and thus to avoid clogging the side tube by the crystals forming on cooling the distillate. The vapors were allowed to pass two receivers, the first consisting of an ordinary distilling flask cooled with running water. This flask in its turn was connected with a U-tube which was cooled by a mixture of solid carbon dioxide and acetone.

The distillation was at first conducted without special precaution until the temperature of the bath reached 70°, at 15 mm. pressure. In this manner all the residual alcohol as well as some volatile acids and some methylglyoxal were removed. When this temperature was reached the above described system was introduced and the distillation then proceeded at very low pressures. Three fractions were collected at 0.2 to 0.6 mm. pressure and at the temperature of the vapors from 93–110° (120–130° of the bath). The more volatile material collected in the U-tube, the less volatile in the first receiver, and finally the residue in the original distilling flask.

(*c*) *The More Volatile Fraction of Extract I.*—This portion consisted chiefly of volatile fatty acids, perhaps principally of formic acid, and of methylglyoxal. The acids have not been identified. The latter was identified as its phenylosazone which was prepared in the usual way. It was recrystallized from hot alcohol and washed with cold alcohol repeatedly. It then melted at 145° and had the following composition.

3.365 mg. substance: 0.645 cc. N_2 at 764 mm. and 24°.

$C_{15}H_{16}N_4$.	Calculated.	N 22.21.
	Found.	" 22.23.

The distillate gave with Schiff's reagent a red coloration on standing and when heated to boiling it developed yellowish green vapors which are characteristic of methylglyoxal.

(*d*) *The Less Volatile Fraction of Extract I.*—The distillate collected in the first receiver consisted of a thick syrup filled with a mass of crystals. The mass was extracted in the cold first by a solution of alcohol and ether (1:1) and then by acetone.

The crystals had a very sharp melting point at 82°. The reducing power toward Fehling's solution was about 60 per cent of that of glucose. The substance formed a phenylosazone in the cold and readily formed a bisulfite derivative. It had the elementary composition of $C_3H_6O_3$ as is seen from the following data. The molecular weight determined cryoscopically in water showed its monomeric composition.

15.1584 gm. H_2O, 0.2907 gm. substance; 0.407° freezing point depression. Found, molecular weight 87.5.

15.1584 gm. H_2O, 0.5047 gm. substance; 0.707° freezing point depression. Found, molecular weight 87.5; calculated, 90.0.

The substance had the following composition.

4.675 mg. substance: 6.920 mg. CO_2 and 2.860 mg. H_2O.
$C_3H_6O_3$. Calculated. C 40.00, H 6.66.
Found. " 40.36, " 6.84.

(*e*) *Distillation Residue* (*18.5 Gm.*) *of Extract I.*—This was apparently a very complex mixture. *En masse*, it analyzed for $C_6H_{10}O_5$, that is, for a substance formed from 2 molecules of dihydroxyacetone through loss of water, as seen from the following analytical data.

0.1030 gm. substance: 0.1700 gm. CO_2 and 0.0566 gm. H_2O.
$C_6H_{10}O_5$. Calculated. C 44.42, H 6.17.
Found. " 45.00, " 6.14.

In fact, it was easily proved that the material was a mixture of condensation products with the monomeric dihydroxyacetone or possibly with a true dimeric form. The hydroxyacetone tenaciously adhered to the condensation product.

The product was acetylated in the following way. To 5 gm. of the brown-colored residue were added 30 cc. of acetic anhydride and the mixture was refluxed for 2 hours. The excess of anhydride was removed by distillation at 15 mm. pressure. The distillation was then continued at 0.1 to 0.2 mm. pressure. At 85° 1.4 gm. of crude diacetyldihydroxyacetone distilled over. The distillate crystallized in the receiving flask in long needles. The crystalline material was recrystallized from ether and petrolic ether and the crystals were then dried on hardened filter paper. The substance had the same melting point as recorded by Dimroth and Schweizer,[5] namely 46–47°. It analyzed as follows:

5.815 mg. substance: 10.320 mg. CO_2 and 2.965 mg. H_2O.
$C_7H_{10}O_5$. Calculated. C 48.28, H 5.75.
Found. " 48.39, " 5.70.

The distillation residue showed the composition of a condensation product.

4.645 mg. substance: 8.670 mg. CO_2 and 2.325 mg. H_2O.
Found. C 50.9, H 5.6.

[5] Dimroth, O., and Schweizer, R., *Ber. chem. Ges.*, 1923, lvi, 1375.

(*f*) *Fractionation after Preliminary Extraction with Ether.*—A portion of the concentrate of Extract I was dissolved in very little water and extracted with ether for 30 hours. The residue was concentrated under reduced pressure to remove the water and then distilled at 0.2 to 0.5 mm. pressure. At the temperature of the bath of about 120–125°, a distillate came over consisting of the monomeric dihydroxyacetone. In this experiment the residue did not have the dark brown color which it did in the other experiment, but was only slightly yellow. It had a waxy appearance. This material was acetylated and the acetylation product was distilled. At a pressure of 0.2 to 0.5 mm. and at a temperature of the bath of 110–125°, a fraction came over which had the melting point of diacetyldihydroxyacetone. At the temperature of the vapors of 138–150°, at 0.5 to 0.8 mm. pressure, an oil came over which had the composition of diacetyldihydroxyacetonyldihydroxyacetone. The substance gave the following analysis.

3.490 mg. substance: 6.230 mg. CO_2 and 1.835 mg. H_2O.
$C_{10}H_{14}O_7$. Calculated. C 48.76, H 5.73.
Found. " 48.68, " 5.88.

The molecular weight in benzene by the method of Menzies and Wright was as follows: 32.0 cc. of benzene (b. p. 80.4° at 767 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
gm.	*mm.*	
0.1294	9.2	287
0.2949	20.8	289

$C_{10}H_{14}O_7$. Molecular weight calculated, 246.

2. Residue I.—This residue is the material remaining after extraction of the crude material with alcohol and ether. As stated above, it had the character of a slightly soft solid. The reducing power of the substance toward Fehling's solution in the cold was equivalent to 40 per cent of that of glucose. The material treated with phenylhydrazine in the cold formed an osazone nearly instantaneously, but the quantity of osazone formed continually increased. For analysis it was recrystallized from alcohol. Its composition was as follows:

3.240 mg. substance: 0.596 cc. N_2 at 764 mm. and 25°.
4.465 " " : 11.075 mg. CO_2 and 2.300 mg. H_2O.
$C_{15}H_{16}ON_4$. Calculated. C 67.13, H 6.00, N 20.88.
Found. " 67.64, " 5.76, " 21.10.

3. Extract II.—Residue I was covered with acetone and thoroughly ground in a mortar, the acetone being renewed until the soft solid turned into a dry powder (Residue II). The extracts were combined, filtered, and concentrated under reduced pressure at room temperature. A thick syrup was obtained in this manner much more viscous than Extract I. This syrup was then distilled at 0.2 to 0.6 mm. pressure and at the temperature of 93–110°. The distillate was treated exactly as that in the case of Extract I. From it monomolecular dihydroxyacetone was obtained, with the same properties as the corresponding fraction of Extract I. The residue had a different appearance from that of the corresponding fraction of Extract I. It was lighter in color and had a harder consistency. After a preliminary test it was found most convenient to take the mass up in warm dilute methyl alcohol. The greatest part dissolved, leaving a certain amount of undissolved crystals. To this suspension an equal volume of acetone was added and the suspension was allowed to stand overnight and filtered. This substance was found insoluble in water, in alcohol, in acetone, in pyridine, in glacial acetic acid, and in acetic anhydride. It was soluble in dilute aqueous alkali. The melting was sharp at 252°. The reducing power for Fehling's solution was 11.10 per cent of that of glucose in the cold and 22.72 per cent on warming. Its composition was C 45.5, H 5.91. For further purification the crystalline material was exhaustively extracted with acetone. Through this purification the melting point was raised to 258°. The solubility was not altered, nor was the reducing power. The composition of the substance was the following.

6.360 mg. substance: 10.750 mg. CO_2 and 3.385 mg. H_2O.

$C_9H_{14}O_7$.	Calculated.	C 46.15, H 5.98.
	Found.	" 46.09, " 5.95.

Thus the substance seems to be composed of 3 molecules of dihydroxyacetone with the loss of 2 molecules of water.

4. Residue II.—This is the residue obtained from the crude material by extraction with a solution of equal parts of alcohol and ether, and subsequently with acetone. It was a dry powder with the same solubilities and the same reducing properties as Residue I. However, it no longer formed an insoluble osazone

as Residue I did, perhaps owing to the fact that it contained only a little of the uncondensed dihydroxyacetone. This residue was soluble in hot methyl, ethyl, and butyl alcohols and in pyridine at room temperature. The substance contained 42.83 per cent of carbon and 5.93 per cent of hydrogen.

3.0 gm. of this substance were dissolved in pyridine, 2 volumes of ether were added, and the solution concentrated, under reduced pressure (12 mm.), nearly to dryness. The residue consisted of a syrupy mass imbedded with crystals. The crystals were freed from the mother liquor by means of a solution of equal parts of alcohol and ether. The crystalline material obtained in this manner melted sharply at 164°. The reducing power was 75 per cent of that of glucose. It did not form an insoluble phenylosazone, nor did it form a bisulfite derivative. The substance analyzed correctly for $C_6H_{10}O_5$ as seen from the following data.

5.680 mg. substance: 9.240 mg. CO_2 and 3.340 mg. H_2O.

$C_6H_{10}O_5$.	Calculated.	C 44.42,	H 6.17.
	Found.	" 44.36,	" 6.59.

When the experiment was performed on a larger scale a substance was obtained of lesser purity having only 43.8 per cent of carbon. Some of this material was acetylated as follows: To 1.5 gm. were added 8 cc. of freshly distilled acetic anhydride, the mixture was refluxed for 1 hour, and the excess of anhydride was removed by distillation at 15 mm. pressure. The acetylation product was distilled at 0.1 to 0.2 mm. pressure and at a temperature of 83–85°. The distillate consisted of diacetyldihydroxyacetone which crystallized on cooling. The crystals had a melting point of 46–48°. The residue consisted of diacetyldihydroxyacetonyldihydroxyacetone as seen from the following analytical data.

7.270 mg. substance: 13.120 mg. CO_2 and 3.750 mg. H_2O.

$C_{10}H_{14}O_7$.	Calculated.	C 48.76,	H 5.73.
	Found.	" 49.21,	" 5.77.

THE EFFECT OF INSULIN AND OF PANCREATECTOMY ON THE DISTRIBUTION OF PHOSPHORUS AND POTASSIUM IN THE BLOOD.

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INTRODUCTION.

The simultaneous fall in the concentration of glucose and of inorganic phosphate in the blood after the administration of insulin, together with the discovery by Embden and his coworkers (1–3) of hexose phosphoric esters in striated muscle, has led a number of investigators (4, 5) to suggest that the function of insulin may be to cause a combination of the glucose and phosphoric acid to form an ester similar to or identical with Embden's lactacidogen.

The organic acid-soluble phosphorus compounds which are present in blood corpuscles in considerable quantity have been shown by Kay and Robison (6) to consist of at least two phosphoric esters, one of which is readily hydrolyzed by an enzyme present in ossifying cartilage. The possibility that one of these compounds might be a hexose phosphoric ester, possibly the lactacidogen of muscle, was tested by Kay and Robison (6). They attempted to observe whether any hydrolysis of the esters occurred in the presence of muscle enzymes, which are known to hydrolyze at least one of the phosphoric acid groups of the diphosphoric ester. The results were inconclusive, and failed to prove the presence of the hexose diphosphoric ester in normal blood. Other experiments however, on the effect of insulin on the distribution of phosphorus compounds in blood, led them to conclude that insulin causes the synthesis of esters of phosphoric acid in the red blood corpuscles from the glucose and inorganic phosphate of the blood. The possibility that the esters thus synthesized may be identical with one of the hexose phosphates found in muscle by Embden makes further study of this problem desirable. Experiments conducted in this laboratory have failed however to confirm the findings of Kay and Robison. In view of the importance of their conclusions, the results of our experiments are presented.

The work of other investigators bearing indirectly on this subject should first be mentioned. Goodwin and Robison (7) isolated from blood a phos-

phoric ester which had reducing properties, was optically active, and was hydrolyzed by the bone phosphatase. They did not state whether or not it may be hydrolyzed by muscle enzymes. Greenwald (8) isolated from blood a non-reducing organic phosphorus compound, possibly a salt of the diphosphoric ester of *l*-glyceric acid. Jost (9) found that the greater part of the organic acid-soluble phosphorus of the corpuscles is in the form of the diphosphoric ester of glyceric acid. Cuthbertson (10) found a decrease in the organic acid-soluble phosphorus of whole blood during fatigue, but failed to record the proportion of red corpuscles, which contain nearly all the organic acid-soluble phosphorus. His findings therefore may have been due merely to a fall in hematocrit. Hynd (11) found that one-sixth of the sugar disappearing from the blood after insulin may be accounted for by conversion to a non-reducing complex stored in the corpuscles, which on hydrolysis again yields reducing substances. Kay and Robison, as already stated, had reported an increase in the organic acid-soluble phosphorus of blood corpuscles after insulin administration. Calculated on the basis of 1 molecule of phosphoric acid to each molecule of glucose, the increase of organic phosphorus accounted for about 40 per cent of the sugar which disappeared from the blood. In view of the important rôle attributed to the corpuscles by their conclusions, it seemed worth while to reinvestigate this question.

The experiments reported in this paper were therefore performed with the purpose of determining whether or not the organic phosphorus of the red blood corpuscles is affected by insulin. The experiments divide themselves into two groups: (1) the effect of insulin on the distribution of phosphorus in the blood of normal dogs; and (2) the changes in the phosphorus distribution caused by removal of the pancreas. If the phosphoric esters of the corpuscles are synthesized by the action of insulin, then the complete withdrawal of insulin from the body might be expected to cause a decrease in these esters.

The concentration of total acid-soluble phosphorus and inorganic and lipid phosphorus in both serum and corpuscles is reported. The distribution of potassium between serum and corpuscles was also investigated, since potassium is known to disappear from the serum simultaneously with inorganic phosphorus and glucose (12, 4).

EXPERIMENTAL.

Dogs were used for the experiments rather than rabbits so that at least two fairly large blood specimens might be taken from each animal. Thus the use of control animals was avoided. Blood

samples were taken by heart puncture after injecting cocaine subcutaneously at the site to be punctured, with the result that pain and struggling were eliminated. The blood was aspirated from the heart into a cylindrical separatory funnel, and there defibrinated by gentle stirring with a glass rod. In two experiments (Nos. 5- and 6-Ins.) lithium oxalate was used to prevent clotting. The use of anticoagulants was avoided because of possible effects on the phosphorus distribution and interference with the potassium determinations. To remove any shreds of fibrin which failed to cling to the glass rod, the blood was filtered through acid-washed gauze directly into 50 cc. centrifuge tubes, with a minimum of disturbance, avoiding excessive loss of CO_2.. Samples of whole blood were immediately taken for determinations of water, hematocrit, lipid phosphorus, and for the preparation of the trichloroacetic acid and tungstic acid filtrates. The rest of the blood was centrifuged at 4000 R.P.M. for 25 minutes. At the end of this period the column of corpuscles had the appearance of laked blood, indicating complete separation of serum from the corpuscles (13). Hematocrit tubes (10 cm. in length) were centrifuged in duplicate together with the blood. The serum was next aspirated as completely as possible into another vessel, and the corpuscles freed from the remaining traces of serum by wiping with strips of filter paper. There was no noticeable hemolysis except for the one case which is noted in the tables.

The serum and corpuscles were each analyzed directly for total acid-soluble phosphorus and potassium. Whole blood and serum were analyzed for inorganic phosphorus, lipid phosphorus, and glucose, the corpuscle composition being calculated from these after determination of the percentage of corpuscles in the whole blood. Water determinations were made on whole blood, serum, and corpuscles.

Since it is difficult to measure accurately samples of corpuscles by volume, it was considered best to weigh the samples of corpuscles, and those of whole blood and serum as well, in order to report all results in terms of mg. per 100 gm. of material rather than per 100 cc. Hence in preparing the tungstic acid, trichloroacetic acid, and lipid filtrates, the samples were first weighed in the volumetric flasks in which the precipitation of protein was to be made.

Analytical Methods.

Water was determined in whole blood, serum, and corpuscles by drying 1 cc. portions at 110° for 24 hours. The samples were measured quickly into small weighing bottles containing cones of fluted filter paper, the bottles and paper having been dried for 24 hours, cooled, and weighed just previous to use.

The *percentage of corpuscles by weight* rather than volume is required for calculating the composition of corpuscles from analyses of whole blood and serum, if results are to be expressed in mg. per 100 gm. This value may be calculated accurately by means of the following formula, as explained in a previous publication (14).

$$\text{Percentage corpuscles} = \frac{100\ (H_2O_s - H_2O_{wb})}{H_2O_s - H_2O_c}.$$

Wherever corpuscle composition was calculated from the analysis of whole blood and serum, the following formula was used:

$$X_c \quad X_s - \left[\frac{100\ (X_s - X_{wb})}{\text{Per cent}_c}\right]$$

where X represents the number of mg. per 100 gm. of the substance in question. The percentages of inorganic phosphorus, lipid phosphorus, and glucose in corpuscles were calculated in this way.

Blood sugar was determined by the method of Benedict (15).

Inorganic, total acid-soluble, and lipid phosphorus were determined by the method of Fiske and Subbarow (16). The precipitation of blood proteins and the determination of inorganic phosphorus were carried out without delay in order to avoid changes in the inorganic phosphorus. The loss of some CO_2 during defibrination undoubtedly renders the blood slightly more alkaline, causing the conversion of a very small amount of inorganic phosphorus into an organic ester (17). The values for organic phosphorus were obtained by subtracting inorganic from the total acid-soluble phosphorus. The corpuscle filtrate was prepared by weighing accurately about 13 gm. of corpuscles in a 200 cc. volumetric flask, laking with water, precipitating the proteins by addition of sufficient 20 per cent trichloroacetic acid to bring the

final concentration up to 8 per cent, and diluting to the mark with water after bubbles ceased to form. Hydrolysis of any of the organic phosphorus during laking introduces no error, since inorganic phosphate is not determined in the corpuscle filtrate, but calculated from whole blood and serum analyses.

Potassium was determined by the method of Kramer and Tisdall (18) with the modifications described by Kerr (14). By use of stronger permanganate and oxalate in the titration of the potassium cobalti-nitrite, the method may be given greater flexibility and permit the determination of potassium over a wider range of concentration than in the original method. The following procedure gave good results.

To the washed precipitate of potassium cobalti-nitrite in the centrifuge tube are added 2 cc. of 0.03 N permanganate and 1 cc. of 4 N sulfuric acid. The tube is placed in boiling water for 1 minute, the precipitate being stirred with a fine glass rod. 2 cc. of 0.025 N sodium oxalate are introduced with a pipette, the contents of the tube stirred until the permanganate is decolorized, and the tube reheated for about 15 seconds. The excess of oxalate is then titrated with 0.03 N permanganate from a micro burette.

The determination of potassium in blood filtrates was accompanied in every case by a control determination of the potassium in an artificial solution of blood salts of known composition.

All analyses for inorganic, total acid-soluble, and lipid phosphorus, potassium, and blood sugar were done in duplicate or triplicate.

In the second group of experiments, 24 hour urine specimens were collected, preserved with toluene, and analyzed for glucose and total nitrogen in order to calculate the dextrose-nitrogen ratios.

Serum proteins were calculated from the refractive indices by the method of Neuhausen and Rioch (19).

Experiments on Overdosage with Insulin.

In order to determine the effects of overdosage with insulin on normal dogs, the normal blood was taken after 18 to 24 hours fasting. Insulin was then injected subcutaneously. The second blood specimen was taken during the period of depression, gener-

TABLE I.

Experimental Details, with Data on Hematocrit and Blood Dilution.

Ins. denotes experiments on the effect of overdosage with insulin on normal dogs; DP., depancreatized dogs.

Experiment No.	Weight of dog.	Time.	Insulin injected.	Blood taken.	Specimen.	H_2O in whole blood.	H_2O in serum.	H_2O in corpuscles.	Corpuscles by weight.	Corpuscles by volume.	Serum proteins.	Remarks.
	kg.	*a.m.*	*units*	*cc.*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
5-Ins.	18.0	8	50	60	Normal.	77.66	90.20	65.19	50.1	47.9		Lactating female, fasted 20 hrs. No signs of depression at 11 a.m. Lithium oxalate used as anticoagulant.
		11		60	After insulin.	78.25	90.77	64.59	47.8	47.0		
6-Ins.	12.5	8	50	65	Normal.	80.77	89.64	66.33	38.1	35.1		Fasted 18 hrs. No signs of depression at 11 a.m. Lithium oxalate used as anticoagulant.
		11		75	After insulin.	82.60	90.42	66.16	32.2	30.6		
7-Ins.	15	7.10	80	80	Normal.	81.70	92.32	65.73	39.9	39.2	7.5	Fasted 19 hrs. Depressed and unable to stand at 9.30 a.m. Defibrinated blood.
		9.45		80	After insulin.	80.98	92.28	66.38	43.6	41.9	7.5	
8-Ins.	15	7.25	80	80	Normal.	81.33	91.72	66.42	41.1	38.8		Depressed and unable to stand at 9.40 a.m. Defibrinated blood.
		9.40		80	After insulin.	81.82	91.98	66.68	40.2	38.2		
9-Ins.	19.5	9	80	90	Normal.	80.97	92.18	66.27	43.3	42.0	7.4	Fasted 22 hrs. Depressed at 11 a.m. Defibrinated blood. Some hemolysis in second sample.
		12.30		90	After insulin.	81.55	92.44	66.74	42.4	40.0	7.1	

10-Ins.	6.5	7.25	70	80	Normal.	78.82	90.32	65.84	47.0	45.4	9.2	Fasted 24 hrs. Dog is uneasy at 9.45 a.m., depressed at 11, muscles twitching at 11.30. Defibrinated blood.
		11	50									
		11.40		110	After insulin.	80.95	91.32	66.66	42.1	41.0	8.1	
17-DP.	19.5	July 18		80	Normal.	79.32	91.67	66.24	48.6	47.8	7.8	Depancreatized July 1. Aug. 5, bled at onset of convulsions, 3 hrs. after the insulin.
		Aug. 4		75	Diabetic.	83.28	93.56	65.78	37.0	35.3	6.1	
		" 5	80	75	After insulin.	84.93	94.12	66.65	33.5	31.8	5.3	
19-DP.	15.5	July 18		90	Normal.	78.07	91.82	65.42	52.1	51.0	7.6	Depancreatized July 20. Aug. 5, bled at onset of convulsions, 1½ hrs. after the insulin.
		Aug. 4		75	Diabetic.	81.07	92.31	65.68	42.2	41.3	7.1	
		" 5	80	75	After insulin.	82.77	93.14	65.83	38.0	36.0	6.5	
22-DP.	8.0	July 19		90	Normal.	79.56	90.65	65.18	43.5	42.1	8.7	Depancreatized July 22. Aug. 11, weak and emaciated. Died after taking the blood specimen.
		Aug. 11		70	Diabetic.	89.57	93.42	65.47	13.8	12.0	6.1	
24-DP.	7.0	July 26		90	Normal.	78.80	90.62	64.71	45.6	43.5	8.7	Depancreatized July 26. Aug. 17 blood taken during period of depression after insulin.
		Aug. 16		75	Diabetic.	81.48	90.37	65.51	35.8	35.0	6.6	
		" 17	120	100	After insulin.	82.26	91.34	66.20	36.1	34.7	8.2	
26-DP.	6.3	Aug. 11		70	Normal.	82.20	91.67	65.83	36.6	35.7	7.7	Depancreatized Aug. 6. Insulin given daily till Aug. 10. "Normal" (post-operative) blood on Aug. 11; diabetic sample Aug. 16. Aug. 17, bled 2 hrs. after insulin, as muscles begin to twitch.
		" 16		75	Diabetic.	83.02	92.26	65.63	34.7	33.5	7.2	
		" 17	70	75	After insulin.	82.67	92.33	67.40	38.7	37.2	7.1	

Experiment No.	Specimen.	Whole blood.				Serum.						Corpuscles.					
		Per cent corpuscles by weight.	Glucose.	Inorganic P.	Lipid P.	Glucose.	Total acid-soluble P.	Inorganic P.	Organic P.	Lipid P.	K	Glucose.	Total acid-soluble P.	Inorganic P.	Organic P.	Lipid P.	K
5-Ins.	Normal.	50.1	88.4		17.0	102.7	5.01	5.00	0.01	16.1	26.5	74.2	59.6	4.76*	54.8	17.9	44.2
	After insulin.	47.8	34.5		16.2	36.5	3.14	2.63	0.51	14.2	20.1	32.3	59.3	3.65*	55.6	18.4	44.3
6-Ins.	Normal.	38.1			13.6		4.41†			12.5	20.3		52.2			15.4	30.7
	After insulin.	32.2			12.8		2.44†			11.8	16.9		52.5			15.0	28.7
7-Ins.	Normal.	39.9	86.6	5.80	14.3	113.5	7.34	6.90	0.44	Lost.	23.6	46.1	57.5	4.14	53.4		33.6
	After insulin.	43.6	42.7	2.47	15.9	47.8	3.73	2.19	1.54	11.5†	14.6	36.1	55.1	2.83	52.3	21.6	38.5
8-Ins.	Normal.	41.1	87.9	5.24	17.8	101.0	6.39	5.86	0.53	20.1	19.4	69.1	51.7	4.35	47.3	14.5	31.5
	After insulin.	40.2	51.3	1.26	17.8	59.3	1.19	1.10	0.09	19.4	8.2	39.4	49.6	1.50	48.1	15.4	29.4
9-Ins.	Normal.	43.3	87.6	5.37	14.9	107.1	7.39	7.03	0.36	15.2	22.5	62.1	59.5	3.20	56.3	14.5	32.7
	After insulin.	42.4	51.3	2.28	15.1	54.2	2.77	2.35	0.42	14.1	12.1	47.4	56.0	2.18	53.8	16.5	33.1
10-Ins.	Normal.	47.0	76.8	3.96	14.6	104.1	4.83	4.63	0.20	11.9	23.0	46.0	51.8	3.20	48.6	17.6	40.8
	After insulin.	42.1	41.8	1.67	13.7	43.9	2.04	1.64	0.40	10.8	17.4	38.9	48.8	1.71	47.1	17.7	38.1
Averages.	Normal.	43.3	85.5	5.09	15.4	105.7	6.19	5.88	0.31	15.2	22.6	59.5	55.4	3.93	52.1	16.0	35.6
	After insulin.	41.4	44.3	1.92	15.3	48.3	2.57	1.98	0.59	14.1	14.9	38.8	53.6	2.37	51.4	16.6	35.4

ally 2 to 3 hours after the insulin injection, before the onset of convulsions. Further experimental details are given in Table I. In this table are also found the values for water content of the whole blood, serum, and corpuscles, the volume hematocrit, the percentage of serum proteins, and the percentage of corpuscles by weight, as calculated from the water determinations. The volume hematocrits were included for the sake of a rough check on the weight hematocrits.

The changes in phosphorus and potassium distribution are found in Table II, which presents the results of the analyses of whole blood, serum, and corpuscles. The values for the corpuscle content of glucose, inorganic phosphorus, and lipid phosphorus were calculated from the figures for whole blood and serum, as already explained.

The results in Table II show the simultaneous fall in the glucose, inorganic phosphate, and potassium of serum, already reported by a number of investigators (4, 12).

The inorganic phosphate of serum decreases greatly after insulin, the total acid-soluble phosphorus falling simultaneously, since it is composed almost entirely of inorganic phosphate. The small amount of organic phosphorus which is found in the serum increases as a result of the insulin in all cases except Experiment 8-Ins. The quantities present are however so small that these results are of doubtful significance.

The greater part of the total acid-soluble phosphorus of blood is the organic phosphorus of the corpuscles, which forms the subject of chief interest in this investigation. The results show practically no change in the total acid-soluble phosphorus in two cases, but a decrease in four cases. When the inorganic phosphorus is subtracted, the organic phosphorus is found to remain practically unaffected by insulin, the average of the six experiments showing a very small decrease. As a check on these direct determinations of total acid-soluble phosphorus in the corpuscle filtrates, determinations were also made on whole blood in four of the six experiments, the corpuscle composition being calculated from the whole blood and serum values. The results agreed within 1 per cent of those obtained by direct analysis. There can be no doubt therefore that insulin has no effect on the total amount of organic phosphorus in the corpuscles of the dog.

After insulin there is a small but consistent decrease in the lipid phosphorus of the serum, with a slight increase in the corpuscles in all but one case. The slight increase in the water content of serum which is noted to occur always after insulin (Table I) is not sufficient to account for the decrease in the lipid phosphorus. The change in the water content is probably due at least in part to dilution of the blood by tissue fluids after withdrawal of the first blood sample.

The concentration of potassium in the serum is greatly decreased after insulin. In the corpuscles the changes are very small; a fall in three cases, and a distinct rise in one (Experiment 7-Ins.), with the average for the six experiments showing no change. In a former investigation (20) experiments on three dogs showed slight increases in corpuscle potassium after insulin. There is no reason to doubt the accuracy of those experiments, but the evidence in the present case indicates without question that the potassium which disappears from the serum does not enter the corpuscles.

Experiments on the Effect of Pancreatectomy, and Subsequent Overdosage with Insulin.

Although it has been shown that even overdosage with insulin does not cause an increase of the organic acid-soluble phosphorus or of the potassium of corpuscles, the possibility remains that a total withdrawal of insulin from the body might cause losses of these compounds from the corpuscles.

A second group of experiments was therefore conducted to determine whether removal of the pancreas would result in changes in the distribution of phosphorus and potassium. A number of dogs were placed on a diet of lean meat (20 gm. per kilo of body weight), which was maintained throughout the experiment. After 10 days on this diet, blood was taken for analysis, this representing the normal period. The pancreas was then completely removed from each dog under ether anesthesia. In order to permit healing of the wounds the animals were given moderate doses of insulin twice daily for 7 to 10 days after the operation. The insulin was then withdrawn and the dogs allowed to become completely diabetic. The D:N ratio was determined in the urine each day after the withdrawal of insulin. After 5 days (11 days

PROTOCOL 1.

Record of Experiments on Depancreatized Dogs.

Experiment No.	17	19	22	24	26
Weight of dog, *kg*	19.5	15.5	8	8	6.3
Sex	Male.	Male.	Male.	Female.	Female.
Daily ration of lean meat, *gm.*	390	310	160	160	126
Meat diet started	July 8	July 8	July 8	July 14	Aug. 7
Normal blood sample taken	" 18	" 18	" 19	" 26	" 11
Depancreatized	" 21	" 20	" 22	" 26	" 6*
Postoperative recovery period (insulin given twice daily)	" 20–29	" 20–29	" 22–29	" 26–Aug. 2	" 6–10
Diabetic period (no insulin)	July 30–Aug. 4	July 30–Aug. 4	July 30–Aug. 11	Aug. 3–16	Aug. 11–16
Diabetic blood sample taken	Aug. 4	Aug. 4	Aug. 11	Aug. 16	Aug. 16
Insulin given (after bleeding, in 3 doses), *units*	30	30	Died.	30	30
Overdosage with insulin, blood sample taken during the insulin shock	Aug. 5	Aug. 5		Aug. 17	Aug. 17
Amount of insulin given, *units*	80	80		120	70

D:N ratios during the diabetic period.

After stopping insulin.	17	19	22	24	26
1st day	3.01	1.46	1.78	0	0.93
2nd "	3.90	3.33	1.90	0	1.98
3rd "	3.09	2.83	1.93	0	2.94
4th "	2.65	1.93	1.53	0	2.00
5th "	2.81	1.80	2.51	0.22	2.47
6th "			1.28	0.33	
7th "			2.26	0.50	
8th "			1.50		
9th "			1.50		
10th "			1.60	1.05	
11th "			2.17	2.04	

* The normal blood specimen for Dog 26 was taken on the 4th day after the removal of the pancreas, insulin having been given daily during this period.

TABLE III.

Effect of Removal of Pancreas and of Overdosage with Insulin on Distribution of Phosphorus and Potassium in Blood of Dogs.

Mg. per 100 gm.

Experiment No.	Specimen.	Whole blood.			Serum.					Corpuscles.				
		Per cent corpuscles by weight.	Glucose.	Inorganic P.	Glucose.	Total acid-soluble P.	In-organic P.	Organic P.	K	Glucose.	Total acid-soluble P.	In-organic P.	Organic P.	K
17-DP.	Normal.	48.6	60.6	5.26	70.8	6.49	6.26	0.23	21.1	49.8	60.5	4.20	56.3	35.9
	Diabetic.	37.0	338.0	5.21	455.0	6.72	6.45	0.27	23.1	139.0	56.9	3.10	53.8	27.5
	Insulin.	33.5	40.6	2.61	28.7	3.08	2.80	0.28	11.0	64.2	57.5	2.23	55.3	26.0
19-DP.	Normal.	52.1	81.7	3.15	95.1	4.05	3.76	0.29	19.6	69.4	47.7	2.59	45.1	29.5
	Diabetic.	42.2	274.7	3.05	379.0	3.94	3.69	0.25	20.4	132.0	50.1	2.17	47.9	28.0
	Insulin.	38.0	49.9	3.58	39.4	3.97	4.00	(0.03)	26.8	67.0	50.0	2.89	47.1	24.8
22-DP.	Normal.	43.5	71.9	3.10	92.4	3.95	3.78	0.17	18.8	45.3	50.5	2.22	48.3	33.4
	Diabetic.	13.8	185.8	3.89	208.2	4.39	4.08	0.31	17.4	45.9	64.6	2.70	61.9	45.4
24-DP.	Normal.	45.6	63.4	3.16	83.7	4.78	4.03	0.75	22.4	39.2	53.6	2.12	51.5	26.4
	Diabetic.	35.8	226.0	3.63	285.4	4.85	4.26	0.59	17.9	166.0	58.2	2.50	55.7	31.3
	Insulin.	36.1	40.2	1.05	40.8	2.11	0.96	1.15	11.8	39.1	55.3	1.21	54.1	32.8
26-DP.	Normal.*	36.6	164.8†	4.51	212.8†	6.18	5.25	0.93	18.7	81.6†	57.0	3.23	53.8	31.9
	Diabetic.	34.7	254.1	4.40	327.4	5.26	5.06	0.20	21.0	116.1	60.3	3.16	57.1	31.8
	Insulin.	38.7	37.7	1.73	38.1	2.58	1.43	1.15	9.5	37.1	62.9	2.21	60.7	32.3
Averages.	Normal.	45.3	69.4	3.84	85.5	5.09	4.62	0.47	20.1	50.9	53.9	2.87	51.0	31.4
	Diabetic.	32.7	255.7	4.04	331.0	5.03	4.71	0.32	20.0	119.8	58.0	2.73	55.3	32.8
	Insulin.	36.6	42.1	2.24	36.8	2.91	2.30	0.61	14.5	51.9	56.4	2.14	54.3	29.0

* Postoperative "normal."

† Not included in averages.

in Experiments 22 and 24) a second sample of blood was taken, this representing the completely diabetic period. After the taking of this second sample, 30 units of insulin were given in three doses during the day. On the following day a large overdose of insulin was administered with the hope of reversing whatever changes had occurred during the diabetic stage. A third sample of blood was taken 2 or 3 hours later, just before the onset of convulsions. Details of the experimental procedure, together with the D:N ratios, are recorded in Protocol 1.

Due to several fatalities it was necessary to include in the experiment one dog (Experiment 26) whose blood had not been analyzed before the removal of the pancreas.

The results of the analyses are given in Table III. The water content of whole blood, serum, and corpuscles, from which the percentage of corpuscles by weight is calculated are found in Table I.

The change from the normal to the severe diabetic condition is marked by a slight but definite *increase* in the organic acid-soluble phosphorus of the corpuscles in all but one of the five dogs studied, Dog 17 showing a decrease. In Experiment 22 the dog received no insulin for 13 days, was extremely weak and emaciated when the second blood sample was taken, and died a few hours later. In this case the increase of organic phosphorus amounts to 28 per cent of the original value. The average increase for the five experiments is 4.3 mg., or about 8.6 per cent.

Large doses of insulin administered to the diabetic dogs cause insignificant changes in the organic phosphorus of the corpuscles. The level of inorganic phosphorus in serum and corpuscles is not significantly changed due to the removal of the pancreas.

The changes in potassium concentration in the corpuscles are not the same in the five experiments. The only striking change is the increase of potassium during the severe diabetic period in Experiment 22. A similar result is observed in Experiment 24, but the reverse occurs in Experiment 17. The conclusion seems justified that the potassium content of corpuscles is not affected directly by the presence or absence of insulin. Although overdoses of insulin cause a fall in the concentration of potassium in the serum (Experiment 19 excepted), the removal of the pancreas produces no consistent changes.

The blood of one human diabetic patient was available for study during the course of these experiments. Determinations of potassium, inorganic phosphorus, and total acid-soluble phosphorus were made before treatment had begun, and again after several days of treatment with insulin, in order to learn whether insulin caused the same changes as those found in the dog. The analyses, presented in Table IV, show that an injection of 40 units of insulin made no appreciable change in the organic phosphorus or in the potassium concentration of the corpuscles. Human corpuscles resemble those of rabbits rather than those of dogs in their potassium content.

TABLE IV.

Effect of Insulin on Organic Phosphorus and Potassium in Blood of a Human Diabetic.

Mg. per 100 gm.

Date.	Remarks.	Serum.			Corpuscles.			
		Glucose.	Inorganic P.	K	Total acid-soluble P.	Inorganic P.	Organic P.	K
July 22	Untreated diabetic.	667	2.72	17.0	45.9	2.86	43.0	345
" 22–28	Insulin daily.							
" 28, 8 a.m.	40 units insulin.							
" 28, 10.15 a.m.	Blood sample taken.		2.22	7.2	45.0	2.27	42.7	357

After injecting the diabetic animals with insulin there is a noticeable increase in the water content of both serum and corpuscles. The changes are, however, not large enough to affect the interpretation of the results in Table III. Serum protein determinations were made because of the information they might give as to the extent of blood dilution by tissue fluids. The serum proteins fall after removal of the pancreas, and decrease still further after injection of insulin (except in Experiment 24). The changes in water content and serum proteins may be due in part to the drawing of fairly large samples of blood for analysis, with subsequent dilution of the blood by tissue fluids. The malnutrition associated with severe diabetes probably also plays a part in

the lowering of the serum proteins (21). The *changes* in hematocrit are of little significance, since on defibrination varying proportions of corpuscles are removed with the fibrin. This of course in no way changes the corpuscle composition. The hematocrit determinations were made solely for the purpose of calculating the corpuscle content of glucose and inorganic and lipid phosphorus, from the analyses of whole blood and serum.

DISCUSSION.

Examination of the results of analysis shows that the organic acid-soluble phosphorus of the corpuscles of the dog is not increased by insulin. On the contrary it increases slightly when insulin is withdrawn. These results are contradictory to those of Kay and Robison (6). It should be pointed out, however, that these authors worked with rabbits, and that the corpuscles of the rabbit differ distinctly from those of the dog in having a higher content of organic phosphate and of potassium. It seems improbable, however, that insulin should have a different effect on rabbit blood than on dog blood. Some possible reasons for the discrepancy between our results and those of Kay and Robison may be mentioned. These authors present the *averages* of eleven experiments, comparing the blood of normal control rabbits with that of others which had received insulin. The organic acid-soluble phosphorus in the blood of different rabbits may, however, vary as much as 12.6 mg. per 100 cc. according to data presented by these same investigators (22). This variation is most probably due to differences in hematocrit, since the organic phosphorus is found almost entirely in the corpuscles, but individual differences in the phosphorus content of the corpuscles of various rabbits may likewise occur, if we may judge by our experience with dogs. Kay and Robison do not give the hematocrits for either group of rabbits. The results of the individual experiments together with hematocrits or hemoglobin determinations such as the one given in their Table VI (6) would be much more convincing.

Secondly, their analyses, as recorded, actually show a decrease of 1 per cent in the organic phosphorus of whole blood after insulin, but this they interpret as an increase of 14 per cent, since the blood volume is said to increase by "some 15 per cent" under

the conditions of the experiment. In view of the fact that the corpuscles contain practically all of the organic acid-soluble phosphorus of the blood, the necessity of reporting the exact hematocrits in each case is again apparent. This was done in a single experiment (Table VI) in which hemoglobin determinations show the extent of blood dilution. Furthermore, the amount of organic phosphorus found after insulin was corrected for blood dilution by multiplying by the factor $\frac{\text{Hb before injection}}{\text{Hb at death}}$. This however gives a true correction only when the diluting fluid is phosphorus-free. The dilution of the blood must be due in part at least to an inflow of tissue fluids, which contain quantities of phosphorus similar to plasma, and hence the corrected figures are too high. The results obtained by Kay and Robison in the one experiment recorded in their Table VI seem nevertheless to show an increase in organic phosphorus in whole blood even when this factor and the blood dilution are taken into consideration.

The assumption by Kay and Robison of a blood dilution of 15 per cent was based on the work of Haldane, Kay, and Smith (23), but these authors state definitely that "the individual variations from this figure are in some cases large." They also conclude that "comparative quantitative determinations in blood before and after the giving of insulin, particularly in the case of substances not equally distributed between the corpuscles and plasma, can only be accepted if this volume change is taken into account."

For these reasons, the conclusions of Kay and Robison in regard to changes in the organic phosphorus of corpuscles must be questioned, although it is possible that the effect of insulin on rabbits may be different from that on dogs, due to the difference in corpuscle composition. Insulin certainly does not increase the organic acid-soluble phosphorus of the corpuscles of dogs, nor can any of the sugar which disappears from blood be accounted for by the synthesis of phosphoric esters in corpuscles from the inorganic phosphorus of blood.

The presence in the corpuscles of a phosphoric ester with reducing properties (7), however, makes it possible that a carbohydrate group is present in its molecule. The observation of Hynd (11) that insulin causes a synthesis of a non-reducing complex in corpuscles which on hydrolysis again yields a reducing sugar is also

significant. It is quite possible that insulin may cause a synthesis of a hexose phosphoric ester in the corpuscles, the blood sugar combining with an organic phosphate compound already in the corpuscles, without increasing the total organic phosphorus; the inorganic phosphorus going at the same time to the tissues. There is, however, no evidence that this is the case, and it seems more reasonable to believe that the phosphoric esters of the corpuscle, even though they may contain hexose groups, are not concerned with the action of insulin.

SUMMARY AND CONCLUSIONS.

1. Experiments are described showing the effect of insulin overdosage on the distribution of inorganic phosphorus, organic acid-soluble phosphorus, lipid phosphorus, and potassium between the serum and red blood corpuscles of the dog. A second group of experiments shows the effect of removal of the pancreas, and subsequent overdosage with insulin, on the organic phosphorus and the potassium of blood.
2. The organic acid-soluble phosphorus content of corpuscles is not significantly altered by overdosage with insulin. It is slightly increased on removal of the pancreas.
3. The changes found in the organic phosphorus of corpuscles do not support the hypothesis that insulin causes a synthesis of phosphoric esters in the corpuscles.
4. Lipid phosphorus is not significantly affected by insulin.
5. The potassium which disappears from serum after insulin does not enter the corpuscles.

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BIBLIOGRAPHY.

1. Embden, G., and Laquer, F., *Z. physiol. Chem.*, 1921, cxiii, 1.
2. Embden, G., and Zimmermann, M., *Z. physiol. Chem.*, 1924, cxli, 225; 1927, clxvii, 114.
3. Embden, G., *Klin. Woch.*, 1927, vi, 628.
4. Harrop, G. A., Jr., and Benedict, E. M., *J. Biol. Chem.*, 1924, lix, 683.

5. Blatherwick, N. R., Bell, M., and Hill, E., *J. Biol. Chem.*, 1924, lxi, 241.
6. Kay, H. D., and Robison, R., *Biochem. J.*, 1924, xviii, 1139.
7. Goodwin, H. W., and Robison, R., *Biochem. J.*, 1924, xviii, 1161.
8. Greenwald, I., *J. Biol. Chem.*, 1925, lxiii, 339.
9. Jost, H., *Z. physiol. Chem.*, 1927, clxv, 171.
10. Cuthbertson, D. P., *Biochem. J.*, 1925, xix, 896.
11. Hynd, A., *Biochem. J.*, 1925, xix, 1095.
12. Briggs, A. P., Koechig, I., Doisy, E. A., and Weber, C. J., *J. Biol. Chem.*, 1923–24, lviii, 721.
13. Ege, R., *Biochem. J.*, 1927, xxi, 967.
14. Kerr, S. E., *J. Biol. Chem.*, 1926, lxvii, 689.
15. Benedict, S. R., *J. Biol. Chem.*, 1926, lxviii, 759.
16. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, lxvi, 375.
17. Martland, M., *Biochem. J.*, 1925, xix, 117.
18. Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlvi, 339.
19. Neuhausen, B. S., and Rioch, D. M., *J. Biol. Chem.*, 1923, lv, 353.
20. Kerr, S. E., *J. Biol. Chem.*, 1926, lxvii, 721.
21. Peters, J. P., Bulger, H. A., and Eisenman, A. J., *J. Clin. Inv.*, 1925, i, 451.
22. Kay, H. D., and Robison, R., *Biochem. J.*, 1924, xviii, 755.
23. Haldane, J. B. S., Kay, H. D., and Smith, W., *J. Physiol.*, 1925, lix, 193.

THE INACTIVATION OF UREASE.

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The hydrolytic enzyme urease, which transforms urea into ammonium carbonate, has received considerable attention since its discovery by Takeuchi (1) in the soy bean. The occurrence, distribution, and preparation of urease, its rôle in the economy of nature, the mechanism by which it functions, the specificity of its action, reaction to foreign bodies, and practical use in laboratory technique, *etc.*, have been subjected to frequent investigations. Complete and exhaustive reviews of the literature on urease have been made by Lövgren (2) and by Oppenheimer (3) and hence will not be given here.

In the present work a comparative study has been made of the inactivating power of various salts on urease, and the possibility of its standardization with mercuric chloride with use of colorimetric methods. In addition an attempt was made to detect mercury in the blood of patients with bichloride of mercury poisoning by means of its toxic action towards urease. This, however, was unsuccessful. In the experiments concerning the effect of various salts on urease, the inactivating agent was added to a standard urea solution to which was then added a measured amount of the enzyme preparation. After incubation at 50° for 5 minutes, the solution was Nesslerized and compared in a colorimeter with a similarly treated urea solution to which no inactivating agent had been added. In case of complete inactivation no color developed upon Nesslerization as the urease was not able to convert any urea into ammonium carbonate. This technique of direct Nesslerization is essentially that developed by Karr (4) for the determination of urea in blood.

EXPERIMENTAL.

Preparation of Reagents.—(*a*) *The urease* used in the experimental work was prepared according to the method of Koch (5) and is essentially a modification of the older glycerol preparations of Jansen (6), Robinson and Oppenheim (7), and Wester (8). About 45 gm. of permutit were shaken with 200 cc. of 2 per cent acetic acid and, after decantation, shaken twice with distilled water. Then 150 cc. of 0.001 N sulfuric acid and 90 gm. of jack bean meal (Eimer and Amend) were added and, after the contents were shaken for an hour, there were added 450 cc. of glycerol. The contents were then poured upon a fluted filter paper. In the course of 2 or 3 days about 250 cc. of a thick, greenish yellow, slightly opalescent filtrate were obtained which was highly active. Even at room temperature this preparation retains practically its full activity for at least 6 months. Just prior to its use the concentrated glycerol solution was diluted 1:10 with distilled water.

(*b*) *The stock urea solution* was prepared by dissolving 0.643 gm. of pure, anhydrous urea in distilled water and diluting to 1000 cc. For the standard 50 cc. of the stock urea solution were diluted to 1000 cc. It was found that the urea in the standard was gradually hydrolyzed on standing at room temperature. Therefore, the stock solution was kept in the refrigerator and fresh standards frequently prepared from it.

(*c*) *The Nessler reagent* was prepared according to the directions of Koch and McMeekin (9).

(*d*) *The standard solutions* of mercuric chloride were prepared by dissolving 13.535 gm. of mercuric chloride in distilled water and diluting to 1000 cc. This gave a solution containing 10 mg. of mercury per cc. Aliquots of this solution were diluted to 100 cc. with water, yielding standards containing various amounts of mercury. All calculations are based on the metallic ion, unless otherwise specified, since the cation is the effective part of the molecule as far as urease inactivation is concerned.

All chemicals used in the experimental work were marked C.P. Baker's Analyzed.

Effect of Mercuric Chloride on Urease.

Into tubes, previously cleaned with a chromic acid cleaning solution (10) and repeatedly rinsed with distilled water, were placed 5 cc. of the standard urea solution. Then 1 cc. of the various mercuric chloride solutions was added to the tubes. To each tube 1 cc. of freshly diluted urease was added and the tubes were incubated at 50° for 5 minutes. The contents were then diluted immediately to 22.5 cc. with distilled water, made up to 25 cc. with Nessler reagent, and mixed by inversion. The Nesslerized solutions were compared in the colorimeter with the standard urea solution to which no mercuric chloride had been added. In those tubes where complete inactivation of the urease had taken place the solutions remained practically colorless except for a faint yellow tinge given primarily by the diluted Nessler reagent. The color gradation from the colorless solutions to the deeply colored ones, where the urea had been completely converted to ammonium carbonate, was very sharp. For simplicity, all colorimetric readings varying not more than 1 mm. of 20 mm., where the standard urea solution was set, are reported as 20 mm. After a few trials the readings, for all practical purposes, can be made merely by inspection. It was found that only a trace of mercury, 0.003 to 0.004 mg., was necessary to inactivate completely 1 cc. of the diluted urease. An experiment is included in which was used an alcoholic extract of the jack bean meal prepared according to the directions of Folin (11), except that the final preparation was diluted 1:10 with distilled water. 1 cc. of the diluted alcoholic urease required 0.002 to 0.003 mg. of mercury for complete inactivation.

A few drops of phosphate buffer are usually added to the urea solutions to aid the action of the urease. Under the above experimental conditions, however, a few drops of Folin's phosphate buffer solution (11) had no effect upon the amount of mercury required to inactivate 1 cc. of the diluted urease, and, therefore, were not added to any of the subsequent experiments. The results have been checked with solutions containing various amounts of urea, thus indicating that the amount of mercury required to inactivate a definite amount of urease is relatively independent of the concentration of the urea. It is obvious that various brands

of jack bean meal may give different results. However, a number of preparations from the same brand and stock of meal gave very consistent results.

Under similar experimental conditions varying amounts of urease were added to standard urea solutions (5 cc.) containing differing amounts of mercuric chloride. As the amount of urease was increased Table I indicates that a proportionate increase in mercury was necessary to inactivate the enzyme completely, and that the concentration of urease can be readily evaluated in terms of mg. of mercury.

According to Sumner (12) pure urease loses its strength rapidly upon dilution with water, but he later showed this was due to

TABLE I.

Mercury Required for Various Quantities of Urease.

Urease.	Mercury required for inactivation.
cc.	*mg.*
1	0.003
2	0.006
3	0.010
4	0.013
5	0.017
6	0.019
8	0.024
1(Concentrated.)	0.035

traces of lead in the distilled water. The glycerol urease used in the above experiments was diluted with distilled water and tested at hourly intervals with the mercury standards and was found to retain its full activity for about 6 hours, after which a gradual deterioration took place.

In a series of experiments, tubes containing 5 cc. of the standard urea solution, mercuric chloride of various amounts, and 1 cc. of urease (diluted) were incubated at 50° and examined at hourly intervals. Results similar to those given in Table I were secured even after an incubation of several hours. However, after 24 to 48 hours at room temperature, a gradual conversion of urea to ammonium carbonate was secured even in the presence of mercuric chloride.

According to various investigators (13–16) the optimum pH for urease is 7.3 to 7.5. In view of the fact that many of the salts used in the experimental work dissociate, yielding acidic or basic reactions, all standard salt solutions were adjusted to a pH of 7 ± 0.3.

Experiments with Blood.

Evidence has recently been presented that during bichloride of mercury poisoning, sufficient mercury may accumulate in the blood stream to inhibit the action of the urease in the determination of the blood urea (17). It seemed desirable to extend this observation in the hope that a simple method for the detection of mercury in the blood could be worked out.

(*a*) To 5 cc. portions of fresh steer blood, containing 2 mg. of potassium oxalate per cc. of blood, were added 1 cc. of the various mercuric chloride solutions and 1 cc. of distilled water to the control blood. The flasks were then shaken with a vigorous circular motion for 5 minutes. Then 40 cc. of 0.12 N sulfuric acid (18) were added to each flask and, after shaking gently for 5 minutes, 5 cc. of 10 per cent sodium tungstate were added. After shaking, they were allowed to stand for 10 minutes and then poured upon the filter paper. To 5 cc. of each filtrate was added 1 cc. of freshly diluted urease; they were incubated at 50° for 5 minutes, Nesslerized, and read in the colorimeter against the urea standard as usual. In (*b*), after the addition of the mercuric chloride to the bloods, the filtrates were prepared according to the original technique of Folin (11). In (*c*), 1 cc. of the diluted urease was added to the bloods containing the mercuric chloride and, after standing at room temperature with occasional shaking for 25 minutes, the filtrates were prepared according to Haden's (18) method, with 0.12 N sulfuric acid. In this case 5 cc. portions of the filtrates were immediately diluted, Nesslerized, and read against the standard urea solution which had been previously incubated with urease. It was found that the sensitivity of the test is greatly decreased in the presence of blood—much more mercury being required to inactivate a definite quantity of urease even when the extra dilution is taken into consideration. Undoubtedly a considerable fraction of the mercury is retained by

the coagulum of blood proteins removed by filtration. The above technique was applied to the blood of a number of patients in various stages of mercury poisoning without at any time the presence of mercury being detected, although they developed a high blood urea and non-protein nitrogen, indicating marked renal damage. Several of the patients subsequently died. The test, therefore, was not sufficiently delicate to detect the presence of the mercury in the blood of patients with bichloride of mercury poisoning.

Relative Effect of Various Salts on Urease.

In the next experiment a comparative study has been made of the inactivating power of various salts on urease. Inasmuch as the inactivation of the enzyme is due primarily to the cation, in order to make the experiments comparable, the concentration of the solutions of the various salts has been based upon the percentage of the metallic ion in the molecule. In a number of tests with certain salts, especially the fluorides, the concentration of the solutions has been based upon the percentage of the negative ion in the molecule. The ions upon which the calculations are based are given in the first column of Tables II and III, and the formulas of the salts used are given in the second column. In each case the solutions were adjusted to a pH of 7 ± 0.3. The experimental technique was similar to that of the previous experiments with mercuric chloride, in each case 5 cc. of the standard urea solution, 1 cc. of the salt solutions of various strengths, and 1 cc. of the diluted urease being incubated, Nesslerized, and read as usual. However, in a number of experiments where a large quantity of the inactivating salt was added to the urea solution, a heavy precipitate resulted upon Nesslerization. In those cases the solution, after incubation, was immediately distilled (11) and the distillate then Nesslerized and compared with a similarly treated urea standard. In each case the control urea solution was set at 20 mm. in the colorimeter.

The data are given in Tables II and III. A reading of 20 mm. indicates that the urea had been completely converted to ammonia even in the presence of the inactivating agent, while the negative sign indicates complete inactivation of the urease, the Nesslerized solutions being colorless. The silver ion was found

to be the most effective inactivating agent—only 0.002 mg. being required to inactivate 1 cc. of the diluted glycerol urease. Mercuric chloride was nearly as active—0.002 to 0.004 mg. being necessary. The other metals such as copper, zinc, cadmium, uranium, gold, *etc.*, then followed in order. The results are in

TABLE II.

Relative Effect of Various Salts on Urease.

Ion.	Formula of salt.	Metallic ion present in 1 cc.								
		1 mg.	0.5 mg.	0.3 mg.	0.1 mg.	0.05 mg.	0.01 mg.	0.007 mg.	0.004 mg.	0.002 mg.
		Colorimeter reading.								
		mm.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
Ag	$AgNO_3$	—	—	—	—	—	—	—	—	—
Hg	$HgCl_2$	—	—	—	—	—	—	—	—	20
"	$Hg(CN)_2$	—	—	—	—	—	—	—	—	20
Cu	$Cu(C_2H_3O_2)_2 \cdot H_2O$	—	—	—	—	—	—	25	20	20
Zn	$Zn(C_2H_3O_2)_2 \cdot 3H_2O$	—	—	—	—	30	20	20	20	20
Cd	$CdCl_2 \cdot 2H_2O$	—	—	—	50	20	20	20	20	20
U	$UO_2(NO_3)_2 \cdot 6H_2O$	—	—	—	20	20	20	20	20	20
Au	$AuCl_3$	—	—	—	20	20	20	20	20	20
Pb	$Pb(C_2H_3O_2)_2 \cdot 3H_2O$	—	50	40	20	20	20	20	20	20
F	NaF	—	20	20	20	20	20	20	20	20
F	$KF \cdot 2H_2O$	—	40	20	20	20	20	20	20	20

The minus signs indicate complete inactivation of urease.

TABLE III.

Relative Effect of Various Salts on Urease.

Ion.	Formula of salt.	Metallic ion present in 1 cc.					
		50 mg.	25 mg.	15 mg.	10 mg.	7.5 mg.	5 mg.
		Colorimeter reading.					
		mm.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
Co	$CoCl_2 \cdot 6H_2O$	—	—	—	—	—	20
Ni	$NiCl_2 \cdot 6H_2O$	—	—	—	—	50	20
Ce	$Ce(NO_3)_4$	—	—	—	40	30	20
Mn	$MnCl_2 \cdot 4H_2O$	—	40	25	20	20	20

The minus signs indicate complete inactivation of urease.

general accord with the findings of other investigators using different methods (19–25). The only effective negative ion found was fluorine which is, therefore, included among the metals. Roe, Irish, and Boyd have recently pointed out that the use of fluorides in too large quantities in the preservation of blood may cause an incomplete recovery of the blood urea by the urease method (26). Among the salts which had little if any inactivating action, even when added to the extent of several hundred mg. per cc. of diluted urease, were potassium oxalate, sodium tungstate, potassium chlorate, potassium sulfocyanate, magnesium sulfate, calcium chloride, lithium acetate, strontium chloride, sodium nitrate, sodium bromide, barium chloride, sodium chloride, *etc.*

It was noticed that, after incubation, in those tubes which contained a sufficient amount of the metallic ion to inactivate the urease completely, a definite white opalescence or flocculent precipitate was visible. This flocculation was not observed in the tubes inactivated with sodium or potassium fluoride, nor in those tubes containing large quantities of the ineffective salts. The urease preparation probably contained some protein which was precipitated by the inactivating metal.

SUMMARY.

1. A method has been suggested for the standardization of urease solutions dependent upon the inactivating action of mercuric chloride.

2. The method was not sufficiently sensitive to detect mercury in the blood of patients with bichloride of mercury poisoning.

3. The inactivating power of various metallic ions on urease was found to be in the following order: silver, mercury, copper, zinc, cadmium, uranium, gold, lead, cobalt, nickel, cerium, and manganese.

BIBLIOGRAPHY.

1. Takeuchi, I., *J. Coll. Agric., Imp. Univ. Tokyo*, 1909, i, 1.
2. Lövgren, S., *Biochem. Z.*, 1921, cxix, 215.
3. Oppenheimer, C., Die Fermente und ihre Wirkungen, Leipsic, 5th edition, 1926, ii, 782.
4. Karr, W. G., *J. Lab. and Clin. Med.*, 1924, ix, 329.
5. Koch, F. C., *J. Lab. and Clin. Med.*, 1926, xi, 776.
6. Jansen, B. C. P., *Chem. Weekbl.*, 1915, xii, 483.

7. Robinson, G. M., and Oppenheim, C. J., *J. Lab. and Clin. Med.*, 1919, iv, 448.
8. Wester, D. H., *Ber. pharm. Ges.*, 1920, xxx, 163.
9. Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, 1924, xlvi, 2066.
10. Fred, E. B., A laboratory manual of soil bacteriology, Philadelphia, 1916.
11. Folin, O., Laboratory manual of biological chemistry, New York and London, 3rd edition, 1923.
12. Sumner, J. B., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 287. Sumner, J. B., and Hand, D. B., *J. Biol. Chem.*, 1928, lxxvi, 149.
13. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 211.
14. Rona, P., and György, P., *Biochem. Z.*, 1920, cxi, 115.
15. Luck, J. M., and Seth, T. N., *Biochem. J.*, 1924, xviii, 1227.
16. Barendrecht, H. P., *Rec. trav. chim. Pays-Bas*, 1920, xxxix, 2.
17. Looney, J. M., *J. Biol. Chem.*, 1926, lxx, 513.
18. Haden, R. L., *J. Biol. Chem.*, 1923, lvi, 469.
19. Van Slyke, D. D., and Zacharias, G., *J. Biol. Chem.*, 1914, xix, 181.
20. Falk, K. G., and Sugiura, K., *J. Am. Chem. Soc.*, 1914, xxxvi, 2166.
21. Wester, D. H., *Biochem. Z.*, 1922, cxxviii, 279.
22. Jacoby, M., *Biochem. Z.*, 1916, lxxvi, 275; 1920, civ, 316; 1922, cxxviii, 80, 89, 95; 1923, cxl, 158.
23. Loeb, L., and Bodansky, O., *J. Biol. Chem.*, 1927, lxxii, 415.
24. Jacoby, M., *Biochem. Z.*, 1916, lxxiv, 107.
25. Hosokawa, T., *Biochem. Z.*, 1924, cxlix, 363.
26. Roe, J. H., Irish, O. J., and Boyd, J. I., *J. Biol. Chem.*, 1927, lxxv, 685.

STUDIES ON THE FOLIN METHOD OF ANALYSIS FOR GLUCOSE IN NORMAL URINE.

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In the course of a series of investigations into physiological sugar determinations several facts were discovered, concerning the Folin[1] method of analysis for glucose in normal urine, which led to modifications improving the method. Benedict[2] called attention to the fact that, as first described,[3] Folin's method failed to show quantitative recovery of glucose added to urine. Folin[4] remedied this defect by two changes in the method. In the first place he substituted oxalic acid for sulfuric acid, explaining that the effect of the stronger acid was to cause some interfering substance to dissolve out of the Lloyd's reagent, and designating the interfering substance as "some calcium salt." Its effect was to prevent the quantitative recovery of added glucose. In the second place, because some of the interfering substance went into solution in spite of the use of oxalic acid, he advocated shaking the Lloyd's filtrate with permutit.

EXPERIMENTAL.

Although Folin stated that permutit had "substantially the same effect if added directly to Lloyd's suspension before filtering, and shaking another 2 or 3 minutes," he gave no experimental data on this point. His statement was confirmed, as shown by the data in Table I. In these tests 1.5 gm. of Lloyd's reagent were mixed with 2 gm. of permutit, and the mixture shaken 4

[1] Folin, O., and Svedberg, A., *J. Biol. Chem.*, 1926, lxx, 405.

[2] Benedict, S. R., *J. Biol. Chem.*, 1926, lxviii, 766.

[3] Folin, O., *J. Biol. Chem.*, 1926, lxvii, 357.

[4] Folin, O., *J. Biol. Chem.*, 1926, lxx, 406.

minutes with the diluted urine and oxalic acid. The liquid was filtered through a quantitative filter, and the rest of the procedure carried out exactly as described by Folin.

The effect was further tried of finely powdering the permutit, and it was found that the powdered permutit gave an increase in

TABLE I.

Mg. of "Glucose" per 100 Cc. of Normal Urine.

	Sample No.	Lloyd's reagent and permutit used together.	Lloyd's reagent and permutit used separately.
Permutit, not powdered.	1	78.1	77.7
		78.5	78.8
	2	70.8	68.4
		71.1	71.4
Permutit, powdered.	1	84.2	83.0
		83.8	83.0

TABLE II.

Mg. of "Glucose" per 100 Cc. of Normal Urine

Untreated Lloyd's reagent with permutit.	Acid-treated Lloyd's reagent without permutit.			Acid-treated Lloyd's reagent with permutit.		
Original urine.	Original urine.	Original urine + 40 mg. glucose per 100 cc.	Error.	Original urine.	Original urine + 40 mg. glucose per 100 cc.	Error.
49.6	46.4	85.6	−0.8	46.7	87.0	+0 3
49.6	46.4	83.4	−3.0	46.5	87.5	+1.0
57.2	50.8	88.0	−2.8	51.6	94.2	+2.6
57.2	51.1	89.4	−1.7	52.5	94.7	+2.2
59.7	53.7	91.5	−2.2	55.6	97.6	+2.0
59.1	54.3	92.5	−1.8	56.0	97.6	+1.6

the reducing value of the urine of 5 or 6 mg. per cent, as shown in Table I.

While any increase in reducing value caused by the unpowdered permutit was probably small, it was thought desirable to try to find a way to eliminate the use of permutit in the determinations. Inasmuch as the interfering substance seemed to be soluble in

acid, a quantity of Lloyd's reagent was treated with concentrated hydrochloric acid, washed, filtered, and dried. The reagent so treated still failed to allow quantitative recovery of glucose added to urine. Treatment of another batch with concentrated nitric acid was also unsuccessful. When, however, Lloyd's reagent was extracted with the two acids in succession, a product was obtained which allowed of quantitative recovery of added glucose without the use of permutit. The procedure was as follows: Place 50 gm. of Lloyd's reagent in a container large enough so that the layer formed is only 2 or 3 cm. deep. Pour in 100 cc. of concentrated hydrochloric acid. Allow to stand 24 hours, stirring every few hours. Dilute with distilled water, filter through a Buchner funnel, and wash with water until the filtrate is free of chlorides. Suck air through the mass until it is nearly dry. Then dry either in air or in an oven at 110°, and powder in a mortar. Treat the product in the same manner with concentrated nitric acid. The double extraction with acids yields a product much lighter in color than the original Lloyd's reagent.

The procedure followed in the analyses was exactly the same as that described by Folin, with the exceptions that the acid-treated Lloyd's reagent was used, and that permutit was not used at all. The results obtained are shown in Table II. It will be noticed that the acid-treated Lloyd's reagent not only allows complete recovery of added glucose, but also gives lower reducing values when used without permutit than are obtained when the untreated reagent is used with permutit.

COMPARATIVE STUDIES OF THE METABOLISM OF AMINO ACIDS.

I. CHANGES IN THE NON-PROTEIN NITROGENOUS CONSTITUENTS OF THE BLOOD FOLLOWING ADMINISTRATION OF AMINO ACIDS.*

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(Received for publication, April 2, 1928.)

Since the discovery of the presence of amino acids in the blood by Delaunay (1) and Van Slyke and Meyer (2) and the subsequent confirmation by Abderhalden (3) and Abel, Rowntree, and Turner (4), attention of students of metabolism has been focused on the behavior of the individual amino acids, components of the protein molecule, in the solutions of the problems which involve the behavior of protein in the organism. Earlier studies, for the most part, were confined to investigations of the changes in the composition of the urine after amino acid administration. Relatively few reports are available in which blood studies by the newer methods of blood analysis were carried out, and, for the most part, these are limited to a few amino acids. In this paper, we present the results of blood analyses after the administration enterally and subcutaneously of glycine, *dl*-alanine, *d*-alanine, *dl*-aspartic acid, *d*-glutamic acid, *d*-lysine, and *d*-arginine. In many cases, a number of these acids have been fed to the same animal, so that to some extent, the factor of individual variability is ruled out. The literature which bears upon our experiments will be discussed later in connection with the consideration of our experimental results.

* The material presented here represents an abstract of part of the thesis presented by Margaret Woodwell Johnston in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the University of Michigan.

EXPERIMENTAL.

The experimental animals were male rabbits maintained upon a standard weighed diet of oats and either carrots or cabbage,[1] which was fed at the end of the 6 or 12 hour experimental period, the time of feeding being always the same throughout any individual experiment. In order to insure an adequate fluid intake, 100 cc. of water were given daily by tube in addition to 100 cc. placed in the cage. On control days, the water was administered at the beginning of the first period of the day. On experimental days half the water was fed at the time of the administration of the amino acid and the remainder was administered 3 hours later.

The amino acids used were glycine, *dl*-alanine, *d*-alanine, *d*-glutamic acid, *dl*-aspartic acid, *d*-lysine, and *d*-arginine. Of these, the glutamic acid and lysine were prepared in this laboratory. In addition, a mixture of amino acids (aminoids from milk) was fed. The amount administered in every case, unless otherwise noted, was equivalent to 0.182 gm. of amino nitrogen per kilo of body weight. For the oral administration, glycine, alanine, and arginine were dissolved in 50 cc. of water, while glutamic and aspartic acids were given as the sodium salts in the same volume of fluid. Lysine dihydrochloride was treated with the theoretical amount of sodium hydroxide to neutralize the hydrochloric acid before administration. In those experiments in which the amino acids were given subcutaneously, each acid was dissolved in as small a volume of water as possible and injected. In order to maintain a constant fluid intake, additional water sufficient to give a total volume of 50 cc. as in the feeding experiments, was introduced by stomach tube.

The animals were placed on the weighed diet several days before the beginning of the experiment. In those experiments in which the same rabbit was used as subject repeatedly, a period of at least 2 weeks, usually more, elapsed between successive experimental periods. On the experimental days, blood was drawn from the marginal ear vein before the administration of the amino acid and again at 3, 6, 12, and usually 30 hours following the adminis-

[1] In a few early experiments the rabbits were fed a diet of milk and cane sugar. This, however, was later changed to the carrot-oat diet.

tration. Potassium oxalate was used as an anticoagulant. The bloods were immediately deproteinized and filtered, and determinations of the distribution of non-protein nitrogen were made by the methods of Folin. The term "undetermined" nitrogen in the tables is used to designate non-protein nitrogen, not urea or amino nitrogen.

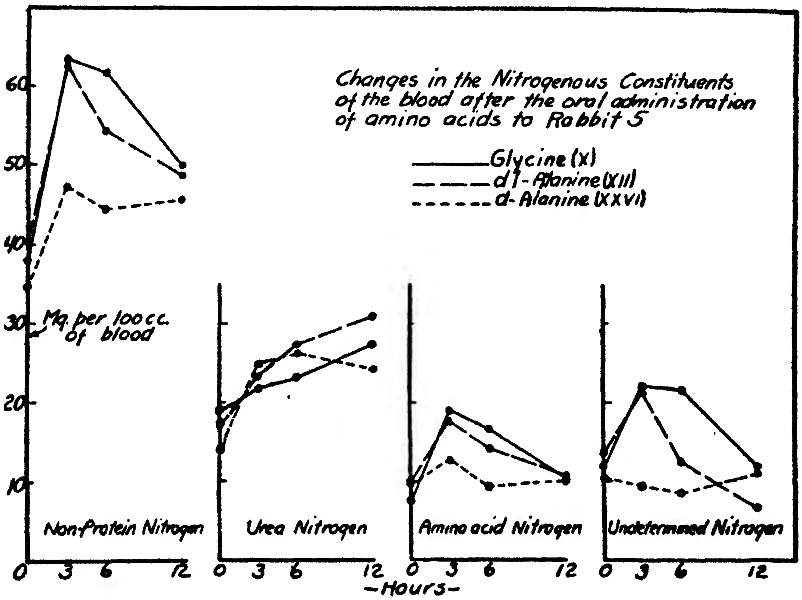

CHART I. The changes in the distribution of non-protein nitrogen of the blood of Rabbit 5, following the oral administration of glycine, *dl*-alanine, and *d*-alanine, equivalent to 0.182 gm. of nitrogen per kilo.

RESULTS.

A. Feeding Experiments.

The results of the experiments with Rabbit 5, in which the entire series of amino acids studied was fed to the same animal, are presented graphically in Charts I and II, in which are shown the variations in the amounts of the nitrogenous constituents of the blood and the time relationships observed. These curves are fairly representative of the typical changes observed in other

feeding experiments as will be evident by a comparison of these results with those of Table I.[2]

Urea Nitrogen.

The maximal increase in the urea nitrogen of the blood at the end of the 3 hour period after oral administration of glycine was

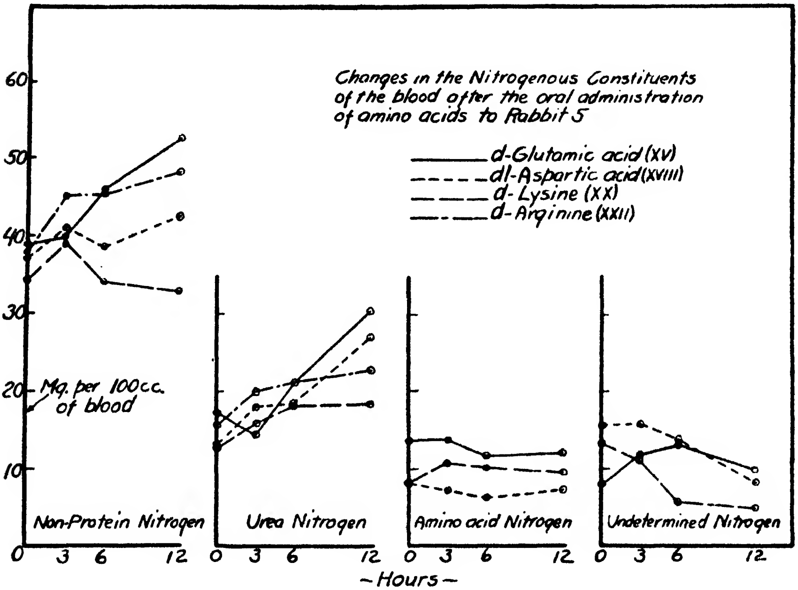

Chart II. The changes in the distribution of non-protein nitrogen of the blood of Rabbit 5, following the oral administration of *d*-glutamic acid, *dl*-aspartic acid, *d*-lysine, and *d*-arginine, equivalent to 0.182 gm. of nitrogen per kilo.

3.6 mg. per 100 cc., an increase entirely comparable with those increases obtained at a similar period after the ingestion of equiva-

[2] With glycine, *dl*-alanine, and *d*-glutamic acid, a very considerable number of experiments, of which those presented are typical, were carried out (seventeen with glycine and nine each with *dl*-alanine and glutamic acid). In the colums headed "Experiment No." of the tables, the arabic numerals indicate the laboratory number of the animals used. It is thus possible to compare results with the same animal after the feeding or injection of different amino acids.

lent amounts of amino nitrogen as arginine and lysine. When, however, *dl*-alanine was fed, the maximal increase in urea nitrogen at this time was greater, 7.4 mg. The maximal increases in urea nitrogen following similar ingestion of *d*-alanine, *d*-glutamic acid, and *dl*-aspartic acid were 10.6 mg., 10.4 mg., and 4.9 mg. respectively. An increase of blood urea nitrogen of 10.4 mg. at the end of 3 hours, after glutamic acid had been fed, did not always occur (compare Chart I), but in a total of seventeen experiments with glycine, in no case was an increase of this magnitude observed as early as 3 hours after feeding. With the exception of this difference in the initial rate of increase in blood urea nitrogen as a result of the ingestion of these various amino acids, the course of the urea nitrogen appears quite comparable, a steady increase, which has reached its maximum value at the end of either a 6 or 12 hour period. The long period elapsing between the collection of the later blood samples does not warrant a more definite statement in regard to the time at which the maximal blood urea nitrogen values are reached.

Amino Acid Nitrogen.

In contrast to the relatively slow increase in the urea nitrogen of the blood following the administration of glycine, an early and marked increase in amino acid nitrogen was observed. In most cases, the value for amino acid nitrogen obtained 3 hours after feeding represented the maximal value observed for this constituent of the blood. It is interesting to note, however, that the amino acid nitrogen of the blood had not returned to its normal fasting level 12 hours after the feeding of glycine. The changes in the amino acid nitrogen of the blood, after *dl*-alanine had been fed, were similar to those obtained after the feeding of glycine, except that usually the maximal value obtained was somewhat lower. When, however, *d*-alanine was fed, either no increase in amino acid nitrogen was observed or if an increase was observed it was slight and transitory.

With one exception, the administration of *d*-glutamic and *dl*-aspartic acid failed to influence the amino acid nitrogen of the blood. In the one exceptional case the maximal increase observed was only 3.1 mg., a slight effect when contrasted with the rises of 10 to 20 mg. observed after the feeding of glycine.

TABLE I.

Changes in the Distribution of Nitrogenous Constituents of Blood Following the Oral Administration of Amino Acids.

All results are expressed in mg. per 100 cc. of whole blood. The amount of amino acid administered was equivalent to 0.182 gm. of nitrogen per kilo of body weight.

Experiment No.	Duration.	Non-protein N.	Urea N.	Amino acid N.	Undetermined N.
	hrs.	*mg.*	*mg.*	*mg.*	*mg.*
III-1. Glycine.	0	54.3	22.1	12.6	19.6
	3	85.8	23.0	21.5	41.3
	6	75.2	22.7	22.9	29.6
	12	66.3	28.7	20.0	17.6
	30	57.8	25.0	15.3	17.5
V-3. Glycine.	0	40.8	15.6	9.2	16.0
	3	76.0	17.9	22.8	35.3
	6	73.4	20.4	23.8	29.2
	12	55.8	19.0	20.7	16.1
	30	40.0	18.1	9.9	12.0
VI-3. Glycine.	0	42.8	19.4	10.1	13.3
	3	74.6	19.9	22.6	32.1
	6	71.4	21.1	19.9	30.4
	12	64.4	23.0	18.5	22.9
	30	50.0	22.6	12.7	14.7
VIII-4. Glycine.	0	41.7	20.0	8.8	12.9
	3	67.8	23.6	22.2	22.0
	6	67.0	29.1	17.8	20.1
	12	56.2	28.6	13.1	14.5
	30	39.7	21.4	9.4	8.9
IV-1. *dl*-Alanine.	0	50.0	22.7	13.5	13.8
	3	73.2	30.1	18.4	24.7
	6	68.8	32.4	19.1	17.3
	12	70.3	37.5	14.5	18.3
	30	57.9	30.0	9.6	18.3
XXVIII-8. *dl*-Alanine.	0	40.8	21.8	8.2	10.8
	3	59.0	27.9	17.9	13.2
	6	53.6	28.3	14.7	10.6
	12	53.0	27.5	11.2	14.3
	30	40.9	22.7	8.3	9.9
XXV-8. *d*-Glutamic acid.	0	39.8	22.8	8.1	9.0
	3	49.8	33.2	8.9	7.7
	6	44.7	32.1	7.2	5.4
	12	46.8	30.4	7.3	9.1
	30	40.0	29.2	7.4	3.4

TABLE I—*Concluded.*

Experiment No.	Duration.	Non-protein N.	Urea N.	Amino acid N.	Undetermined N.
	hrs.	*mg.*	*mg.*	*mg.*	*mg.*
XIII-4. *d*-Glutamic acid.	0	33.6	14.8	7.1	11.7
	3	40.2	17.1	10.3	12.8
	6	41.0	21.1	10.0	9.9
	12	42.7	24.4	6.6	11.7
	30	33.3	14.9	5.9	12.4
XXI-8. *d*-Lysine.	0	37.0	18.1	8.1	10.8
	3	39.8	21.1	11.3	7.4
	6	41.0	20.5	10.7	9.8
	12	42.5	26.7	9.2	6.6
	30	36.0	20.4	8.1	7.5
XXXI-11. Glycine.*	0	38.2	16.5	9.6	12.1
	3	52.1	19.5	17.1	15.5
	6	78.1	21.3	23.8	25.0
	12	59.2	33.5	9.8	15.9
	30	39.1	17.8	8.6	12.7

* Glycine equivalent to 0.182 gm. of nitrogen per kilo was ingested at the usual time and an equal dose 3 hours after the first administration.

The oral administration of lysine (two experiments only) did not markedly affect the amino acid nitrogen of the blood. Our experiments with arginine do not afford much information, since it is known that arginine does not react quantitatively with the reagent employed by Folin. Although no values for the amino acid nitrogen of the blood could be obtained, the changes in the non-protein nitrogen not urea nitrogen of the blood would lead us to believe that the feeding of arginine had little influence on the amino acid nitrogen of the blood.

Non-Protein Nitrogen and Undetermined Nitrogen.

The changes in the non-protein nitrogen of the blood following the feeding of amino acids, except glycine and *dl*-alanine, were such that they could be adequately explained by the simultaneous increases in the urea nitrogen. When, however, glycine was fed at the end of the 3 hour experimental period, a marked increase in non-protein nitrogen was observed which could not be explained satisfactorily by the increases in amino acid and urea nitrogen

observed at the same time. In other words, an increase in undetermined nitrogen of the blood was observed, which in the whole series of seventeen experiments varied from 4.5 to 21.1 mg. per 100 cc. In two of the seven feeding experiments with *dl*-alanine, a definite increase in this undetermined nitrogen also occurred. The maximal increase in the non-protein nitrogen of the blood after the

TABLE II.

Changes in the Distribution of Nitrogenous Constituents of Blood Following the Administration of Mixtures of Amino Acids (Aminoids).

All results are expressed in mg. per 100 cc. of whole blood. The amount of amino acid administered was equivalent to 0.182 gm. of nitrogen per kilo of body weight.

Experiment No.	Duration.	Non-protein N.	Urea N.	Amino acid N.	Undetermined N.
	hrs.	*mg.*	*mg.*	*mg.*	*mg.*
XXXV-11. Aminoids.	0	48.9	20.2	12.1	16.6
	3	55.4	26.4	14.0	15.0
	6	49.8	22.9	10.7	16.2
	12	28.3	23.2	8.9	16.2
XLIV-11. Aminoids.*	0	51.6	22.7	11.7	17.2
	3	60.1	28.0	15.1	17.0
	6	52.0	29.0	11.8	11.2
	12	53.0	30.1	9.6	13.3
	30	41.8	18.6	7.7	15.5
XXXVI-11. Aminoids and glycine.†	0	45.1	20.1	12.1	12.9
	3	71.2	25.5	24.4	21.3
	6	69.0	26.9	20.3	21.8
	12	55.8	28.8	11.9	15.1
	30	47.3	23.3	10.5	13.5

* The amount of nitrogen fed was twice the usual amount.

† The amount of nitrogen fed was twice the usual amount; one-half as aminoid nitrogen and one-half as glycine nitrogen.

ingestion of glycine or *dl*-alanine appeared to occur simultaneously with the maximal amino acid nitrogen value. In the case of the other amino acids studied, the appearance of the maximal rise in non-protein nitrogen was determined by the height of the urea nitrogen fraction.

In the hope of accentuating these striking increases in the undetermined nitrogen, observed after the feeding of glycine, a second

feeding of the usual amount of glycine was given 3 hours after the first. The second administration of glycine further increased the

TABLE III.

Changes in the Distribution of Nitrogenous Constituents of Blood Following the Subcutaneous Injection of Amino Acids.

All results are expressed in mg. per 100 cc. of whole blood. The amount of amino acid administered was equivalent to 0.182 gm. of nitrogen per kilo of body weight.

Experiment No.	Duration.	Non-protein N.	Urea N.	Amino acid N.	Undetermined N.
	hrs.	*mg.*	*mg.*	*mg.*	*mg.*
XXXIV-11. *d*-Glutamic acid.	0	39.3	20.1	11.2	8.0
	3	63.7	26.7	23.6	13.4
	6	64.7	33.4	16.2	15.1
	12	65.3	44.0	9.4	11.9
	30	49.2	33.2	7.8	8.2
XL-16. *d*-Glutamic acid.	0	36.4	15.8	9.7	10.9
	3	65.6	24.0	30.8	10.8
	6	65.6	38.5	14.0	13.1
	12	73.2	57.4	6.4	9.4
	30	50.5	29.8	7.5	13.2
XXXIII-11. Glycine.	0	36.1	18.3	8.3	9.5
	3	58.0	23.6	19.8	14.6
	6	55.7	26.4	14.4	14.9
	12	49.1	28.7	9.6	10.8
	30	43.2	27.3	9.0	6.9
XXXVIII-15. *dl*-Alanine.	0	51.5	21.0	11.4	19.1
	3	59.2	23.0	18.6	17.6
	6	52.2	25.6	13.6	13.0
	12	67.3	26.5	12.1	28.7
	30	43.7	21.1	7.5	15.1
XLV-16. *d*-Alanine.	0	48.5	18.5	9.8	20.2
	3	56.5	28.3	10.3	18.0
	6	47.0	29.2	8.1	9.7
	12	40.2	24.6	6.6	9.0
	30	33.8	15.7	9.0	9.1

undetermined nitrogen significantly in one experiment (Table I) and not in a second. Oral administration of a mixture of amino acids containing an amount of nitrogen equivalent to that usually

fed (aminoids from milk) failed to produce a significant increase in undetermined nitrogen (Table II). When twice the usual amount of nitrogen was fed as aminoids, essentially the same results were obtained. In this connection, it should be pointed out that it is usually stated that the proteins of milk contain no glycine and hence, if glycine is the chief amino acid contributing to the rise in the undetermined nitrogen of the blood, negative results such as we obtained might be anticipated. In further support of this point of view, are results obtained when aminoids and glycine were fed together (Table II), which showed increases in the non-protein nitrogen, amino acid nitrogen, and undetermined nitrogen, similar to those observed when glycine was fed alone.

B. Subcutaneous Administration.

Glycine, *dl*-alanine, *d*-alanine, and *d*-glutamic acid were also injected subcutaneously. With the exception of *d*-glutamic acid this mode of administration produced essentially the same changes as observed in the feeding experiments (Table III). The subcutaneous administration of *d*-glutamic acid was followed by a marked increase in the amino acid nitrogen of the blood obtained 3 hours after the amino acid was injected. This was also accompanied by a marked increase in the non-protein nitrogen and in the undetermined nitrogen.

DISCUSSION.

The results obtained suggest two factors which may possibly influence the rate of metabolism of the amino acids, the rate of absorption from the gastrointestinal tract, and the rate of deamination and subsequent formation of urea. The early and marked increases in non-protein nitrogen and amino acid nitrogen, after glycine and *dl*-alanine were fed, indicate a rapid absorption at a rate not widely different for the two amino acids. Cori (5), in his studies on rats, found that the rates of absorption of glycine and *dl*-alanine from the gastrointestinal tract were 0.048 and 0.045 gm. respectively per 100 gm. of body weight. While it is realized that caution must be exercised in interpreting figures obtained for rats as significant for other species, if these findings

hold approximately for the rabbit, the amounts of glycine and *dl*-alanine used in our experiments should be effectively absorbed within 3 hours, the period at the end of which our first blood samples were taken. The similarity of the results obtained when these two amino acids were administered orally and subcutaneously is additional evidence for the belief that absorption from the gastrointestinal canal in the feeding experiments must have been rapid.

When, however, the dicarboxylic acids, *d*-glutamic and *dl*-aspartic acid, were fed, no significant increase in the amino acid content of the blood was observed and a slow increase in non-protein nitrogen occurred which was accompanied by a proportionate rise in urea nitrogen. That this difference in the curves of non-protein nitrogenous constituents of blood may be due, in part at least, to a slower rate of absorption of the dicarboxylic amino acids from the alimentary canal has been suggested by Seth and Luck (6). Further evidence in support of this view-point is obtained from a consideration of our results following the subcutaneous injection of glutamic acid. In these experiments, the early increases in the non-protein, amino acid, and undetermined nitrogen were entirely comparable to those observed after glycine was administered. It should be noted, however, that both the urea and non-protein nitrogen reached very high levels, which suggested a possible toxic effect with some retention of urea. Complete urine analyses were made in all experiments and in Experiment XL (Table III), only 7 cc. of urine were excreted in the 12 hours immediately following the injection of the glutamic acids, while in Experiment XI (not presented in the tables), a marked although not so striking a decrease in the output of urine occurred. Some evidence of the toxicity of glutamic acid is presented in the literature. Stolte (7) reported the death of a rabbit following the intravenous injection of glutamic acid. Lewis, Dunn, and Doisy (8) have observed some toxic effects accompanied by an oliguria in man after feeding 20 gm. of glutamic acid. In the present series, one animal died within 6 hours after the ingestion of two doses of glutamic acid, administered 3 hours apart. For these reasons, we hesitate to interpret the very high urea nitrogen values obtained in the later periods after injection of glutamic acid as significant and indicative of the extent of deamination of this amino acid.

The only other amino acid which was administered both orally and subcutaneously, *d*-alanine, appeared to be absorbed as readily as were glycine and *dl*-alanine.

If it is assumed that the rate of absorption of glycine and *dl*-alanine are essentially the same, some other factor must be responsible for the fact that, as a rule, the amino acid nitrogen of the blood failed to reach as high a level after alanine was fed as after glycine. This, together with the fact that the rate of increase of the blood urea nitrogen was greater when *dl*-alanine was fed, is suggestive of a difference in the rate of deamination of the two amino acids. The suggestion that, although the rate of absorption from the alimentary canal may be essentially the same for alanine and glycine, the metabolism of glycine may proceed more slowly than that of *dl*-alanine, is further supported by the fact that analyses of the urine fractions, corresponding in periods of time to the collection of blood samples, frequently indicated a depression or at least no increase in urea excretion in the 6 hours immediately following the administration of glycine.

If *d*-alanine, on the other hand, is absorbed as rapidly as are glycine and *dl*-alanine, its rate of deamination must be greater than that of either of these two amino acids, since in all experiments with *d*-alanine, there occurred an early increase in urea nitrogen and no significant increase in amino nitrogen of the blood. It is possible that the differences in the behavior of *dl*-alanine and *d*-alanine may be related to the fact that the latter is the optically active form of the amino acid, which occurs in the living organism.

Our data are also of interest as far as concerns the time relationships of the maximal values for amino acid and urea nitrogen. Whenever increases of amino nitrogen of the blood were observed, these always preceded the maximal increase of urea nitrogen of the blood, regardless of whether the amino acids were administered *per os* or subcutaneously. This is in agreement with the results of Folin and Berglund (9) in man, of Seth and Luck (6) in dogs and rabbits, and of Lewis and Izume (10) in rabbits, but contrary to the results of Van Slyke (11) and of Morgulis (12) in dogs. Both of these investigators, who, however, fed proteins rather than amino acids, observed that the increase in urea nitrogen may precede the increase in amino acid nitrogen. It is well to consider carefully the suggestion of Fearon (13) that the dog

may differ from other animals in that the processes of deamination and urea synthesis are not histologically separated to the same extent, but may be chiefly confined to one organ.

In an attempt to determine the origin and significance of the rises in undetermined nitrogen observed in our experiments with glycine, we have attempted to apply the method suggested by Swanson (14) for the determination of peptide nitrogen in Folin-Wu blood filtrates. The method proved to be so unsatisfactory for the small amounts of blood which were available in our experiments that our failure to observe significant changes in peptide nitrogen cannot be considered as excluding the presence of this type of nitrogenous compound in the blood.

Swanson (14) considered that the peptide nitrogen of human blood accounted for the greater part of the undetermined (rest) nitrogen, while Blau (15) was unable to demonstrate significant amounts of peptide nitrogen in normal human blood. Kalmykoff (16) was unable to determine the presence of peptide nitrogen in dialysates of fasting blood from different blood vessels of a dog, but as absorption of protein digestion products from the gastro-intestinal canal began, polypeptide nitrogen appeared in the blood. He observed also that this polypeptide nitrogen of the portal blood was less than that of the hepatic blood, while in the case of the amino nitrogen of the bloods, the conditions were reversed. He suggested a synthesis of polypeptides from amino acid in the liver. Kotschneff (17), in a continuation of this work, after the introduction of various amino acids (*dl*-leucine, *dl*-valine, "rectamin") into the duodenum of the dog, observed that the normal value of peptide nitrogen (3 to 4 mg. per 100 cc. of blood) was increased to 8.1 to 9.9 mg. in femoral blood, with values of 11.4 to 12.5 mg. in portal blood. This would indicate synthesis of peptides from amino acids in the organism of the dog. Kotschneff considers that the synthesis may take place, not only in the liver, but also in the kidney and muscle as well, since more polypeptide nitrogen was found in the blood from the renal and femoral veins than in arterial blood, after the injection of amino acids into the jugular vein. On the other hand, Van Slyke (11) could find no evidence of an increase in peptide nitrogen in any of the tissues examined following the intravenous introduction of protein hydrolysates to dogs.

Folin and Berglund (9) and Wu (18) have shown that the greater part of the undetermined nitrogen of the blood is contained in the corpuscles. Wu (18) suggests that until further evidence is obtained, the unknown fraction of the nitrogen may be considered as peptide and peptone nitrogen. We consider that the results of these investigators are of significance in relation to our own results since the methods of deproteinization and amino acid determination used by them were the same as those used in the present investigation. Since the various protein precipitants are known to differ in respect to the amount of non-protein nitrogenous material remaining in the filtrate (19), uniform results for the undetermined nitrogen of the blood might not be obtained if different protein precipitants were employed. Abel, Pincoffs, and Rouiller (20) earlier observed that albumoses could be isolated from all the tissues of the body including the formed elements of the blood. They also isolated larger amounts of albumoses from the mucosa of the gastrointestinal tract after a protein meal than in fasting, but did not believe that these substances could be traced from the gastrointestinal tract to the other organs of the body, unless the cellular elements of the blood could be considered as the distributing agents, a conclusion which they felt was not justifiable at that time.

The persistent increases in undetermined nitrogen which we observed after the administration of glycine suggest the possibility of the occurrence of peptide nitrogen in increased amounts in the blood. Why peptide synthesis should occur from glycine and not from the other amino acids studied is not evident. It should be noted, however, that high values for undetermined nitrogen were always associated with a high level of amino acid nitrogen, and that this amino acid nitrogen rose to higher levels following the administration of glycine than of the other amino acids with the exception of glutamic acid subcutaneously injected. In this experiment a high value for the undetermined nitrogen was also observed.

It should also be pointed out that the values for undetermined nitrogen are obtained by difference, and that they are therefore subject to the errors inherent in the other methods of determination. Moreover, little data are available in regard to the manner in which the Folin amino acid reagent reacts with peptides. In

unpublished observations by R. H. Wilson of this laboratory, it was determined that the peptide, diglycyl-cystine, reacted with the Folin reagent to give a value for free amino nitrogen of approximately 50 per cent of the theoretical value. If other peptides react thus in part with their free amino groups in Folin's method, then the values reported by others as well as ourselves for amino acid nitrogen of the blood must be high, including a part of the free amino nitrogen of the peptide. This suggests that our undetermined nitrogen values may be too low and that a part, at least, of the peptide nitrogen, if such exists in blood, is included in the amino acid nitrogen fraction. The parallelism between our high values for amino acid nitrogen and the undetermined nitrogen is at least suggestive and worthy of further investigation. Further work along these lines is in progress.

SUMMARY.

1. The changes in the non-protein nitrogen, urea nitrogen, amino acid nitrogen, and undetermined nitrogen of the blood of the rabbit after the oral and subcutaneous administration of a number of amino acids have been studied.

2. The results suggest that two factors, at least, may influence the rate of metabolism of ingested amino acids, the rate of absorption from the intestine and the rate of deamination in the organism proper. It seems probable that in the rabbit, under our experimental conditions, alanine and glycine are absorbed readily and more rapidly than glutamic acid, but that the deamination of glycine may proceed more slowly than the deamination of the other amino acids studied.

3. A significant increase in the "undetermined" nitrogen of the blood was observed regularly after the administration of glycine and occasionally after *dl*-alanine. The possibility that this may be due to a synthesis of peptide from the amino acid is considered.

BIBLIOGRAPHY.

1. Delaunay, H., *Compt. rend. Soc. biol.*, 1913, lxxiv, 641, 766.
2. Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399.
3. Abderhalden, E., *Z. physiol. Chem.*, 1913, lxxxviii, 478.
4. Abel, J., Rowntree, L. G., and Turner, B. B., *J. Pharmacol. and Exp. Therap.*, 1913–14, v, 275.

5. Cori, C. F., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 125.
6. Seth, T. N., and Luck, J. M., *Biochem. J.*, 1925, xix, 366.
7. Stolte, K., *Beitr. chem. Physiol. u. Path.*, 1904, v, 15.
8. Lewis, H. B., Dunn, M. S., and Doisy, E. A., *J. Biol. Chem.*, 1918, xxxvi, 9.
9. Folin, O., and Berglund, H., *J. Biol. Chem.*, 1922, li, 395.
10. Lewis, H. B., and Izume, S., *J. Biol. Chem.*, 1926–27, lxxi, 33.
11. Van Slyke, D. D., *The Harvey Lectures*, 1915–16, xi, 146.
12. Morgulis, S., *J. Biol. Chem.*, 1925, lxvi, 353.
13. Fearon, W. R., *Physiol. Rev.*, 1926, vi, 399.
14. Swanson, W. W., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 193.
15. Blau, N. F., *J. Biol. Chem.*, 1923, lvi, 873.
16. Kalmykoff, M. P., *Arch. ges. Physiol.*, 1924, ccv, 493.
17. Kotschneff, N. P., *Arch. ges. Physiol.*, 1926, ccxiv, 343.
18. Wu, H., *J. Biol. Chem.*, 1922, li, 21.
19. Hiller, A., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liii, 253.
20. Abel, J. J., Pincoffs, M. C., and Rouiller, C. A., *Am. J. Physiol.*, 1917, xliv, 320.

THE COMPLEX NATURE OF VITAMIN B AS FOUND IN WHEAT AND CORN.*

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Several investigators have made a study of the vitamin B content of the wheat grain but there seems to be a diversity of opinion as to the quantity present. McCollum and associates (1) found 15 per cent of whole wheat in the diet sufficient to supply the necessary amount of vitamin B for normal growth, reproduction, and nursing of the young, but not sufficient to enable the second generation to develop normally. They later concluded that when 33 per cent of wheat in the diet was used as the sole source of vitamin B, it was doubtful whether sufficient vitamin B was present for the rearing of the young. Osborne and Mendel (2) also came to the conclusion that 15 per cent of whole wheat in the diet supplies sufficient vitamin B for normal growth. They state that this is very near the minimum, for with 10 per cent the rats ceased to grow, but recovered when 20 per cent of the diet was whole wheat. Guest and associates (3) found that 10 per cent of wheat contains sufficient vitamin B for normal growth of rats, but that when the amount was increased to 15 per cent one female gave birth to six litters in 10½ months. Bell and Mendel (4) found that spring wheat contains a larger amount of vitamin B than winter wheat and that 15 per cent of spring wheat or 40 per cent of winter wheat was necessary to supply sufficient vitamin B for normal growth. Croll and Mendel (5) showed that corn does not differ greatly from wheat in its vitamin B content, and that the minimum percentage which furnishes an adequate amount for normal growth lies between 20 and 30 per cent of the corn in the diet.

This seemingly high vitamin B content of wheat and corn was no doubt due to the method, in vogue at that time, of keeping the rats on shavings as bedding. In the light of our present knowledge regarding the vitamin B content of the excreta of animals this accounts for the seeming discrepancies. Steenbock and associates (6) kept their rats in screen bottom cages and found that 60 per cent of grains must be in the diet in order to furnish sufficient vitamin B for normal growth.

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During the last 5 years a study has been made in this laboratory of the influence of fertilizers on the vitamin B content of the wheat grain. In this study the rats that were used as the experimental animals were kept in cages equipped with screen bottoms to prevent coprophagy. The data (7) invariably show that the wheat grain is very low in what was originally designated as vitamin B or a vitamin essential for growth. Rats required approximately 65 per cent of whole wheat in the diet to produce normal growth. At this level only 50 per cent normal reproduction occurred, which however was markedly improved by administration of 0.25 gm. of dry powdered yeast to each animal daily. Data were also obtained which showed that the bedding and excreta were equivalent to 25 per cent of wheat in vitamin B content.

Throughout the study peculiar symptoms appeared such as ophthalmia, loss of hair, lesions around the mouth and on the feet and bodies. These symptoms occurred on diets containing 40 per cent or less of wheat. Not all animals developed these symptoms to the same degree—the males seemed to be more affected than the females. These lesions could be prevented or cured and the animals made to grow by feeding dry yeast, either separately or mixed in the diet. Since the symptoms of polyneuritis were not observed in any of the rats receiving as low as 15 per cent of wheat in the diet and since dry yeast induced growth and prevented or cured the above symptoms, the obvious conclusion was that the wheat grain contains the antineuritic vitamin and only a small amount of another vitamin which in conjunction with the antineuritic factor induces growth. The symptoms described above are suggestive of pellagra, which has been described recently by Goldberger and associates (8) and Chick and Roscoe (9). This growth-producing vitamin, which at the same time prevents or cures these pellagra-like symptoms is probably the same as the P-P factor mentioned by Goldberger (8) and what Sherman and Axtmayer (10) choose to call vitamin G.

The data presented in the work mentioned above were rather limited as far as showing the nature of vitamin B and it was desired to obtain further information in regard to the nature of this factor in wheat and extend it to include corn.

Before the investigation mentioned above was begun Emmett and Luros (11), Funk and Dubin (12), and Levene and Muhlfeld

(13) presented some evidence that vitamin B is made up of two factors. While this study was in progress Kinnersley and Peters (14), Hauge and Carrick (15), Smith and Hendrick (16), Goldberger and associates (17), and finally Salmon (18, 19), Chick and Roscoe (9), and Sherman and Axtmayer (10) offered further evidence that vitamin B is composed of at least two fractions.

To avoid confusion the terminology, as suggested by Sherman and Axtmayer (10) has been adopted for this paper; *viz.*, the antineuritic factor is called vitamin F and the growth or antipellagra factor, vitamin G.

EXPERIMENTAL.

The experiments described in this paper were conducted according to the quantitative method of Sherman and MacArthur (20)

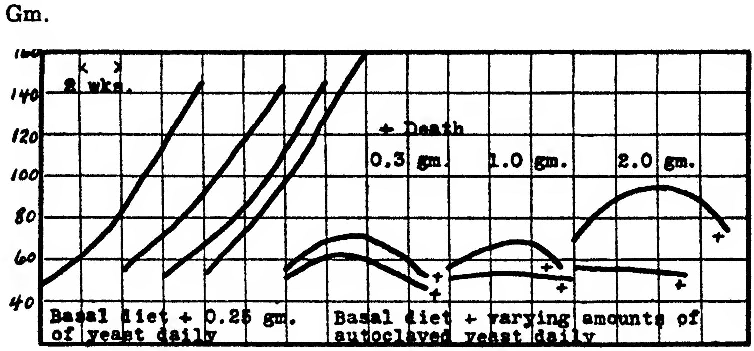

CHART 1.

and by the method of this laboratory (7). Rats 24 days of age and weighing generally from 50 to 60 gm. were placed in cages provided with screen bottoms and given a vitamin B-free diet. The diet contained casein 19, starch 63, McCollum's Salt Mixture 185 4, Crisco 10, agar 2, and cod liver oil 2 parts. The supplements used were wheat, corn, and autoclaved yeast, which were fed separately, daily, and in varying amounts.

The wheat and corn used were grown on the station farm. The grains were cleaned and then ground through a rather fine mesh. The yeast used was obtained from the Northwestern Yeast Company. It was placed in enamel pans to a depth of about ½ inch and autoclaved for 4 hours at 15 pounds of steam pressure.

The weight curves are shown in Charts 1 to 3. It will be seen that an increasing amount of autoclaved yeast had no effect on the rate of growth of the animals. Since 0.25 gm. of the original

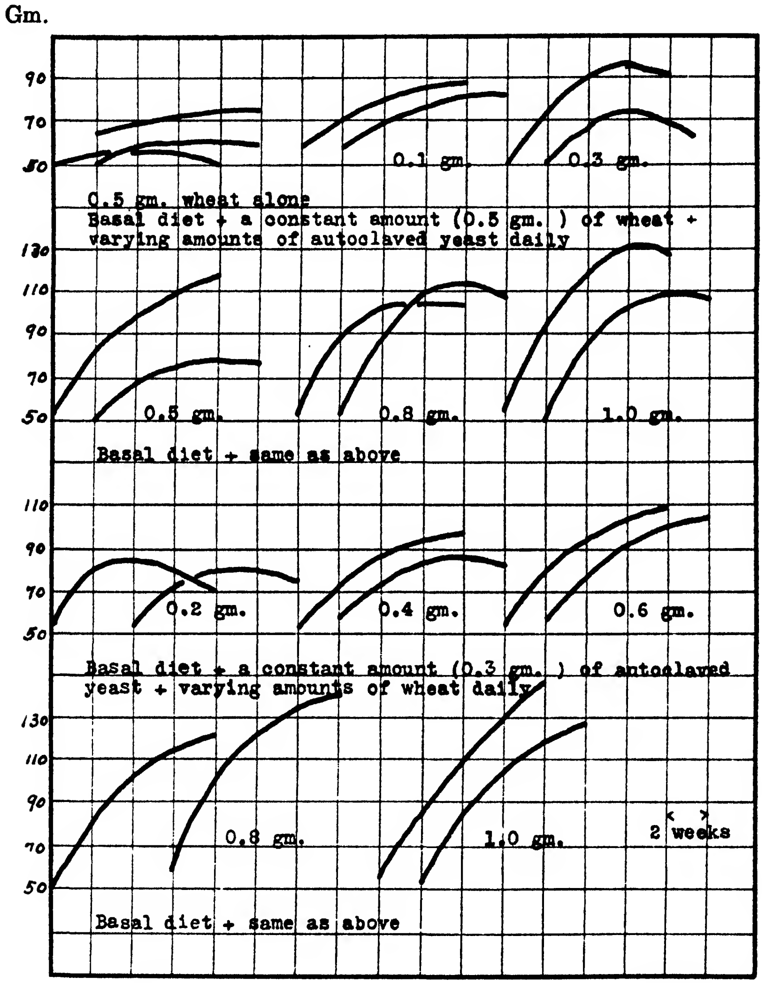

Chart 2.

yeast fed daily supplied sufficient vitamin B for good growth (Chart 1) of rats and that as high as 2.0 gm. of autoclaved yeast failed to produce growth and almost all died of symptoms indica-

tive of polyneuritis, it indicates that in autoclaving the yeast most of the vitamin F (antineuritic) was destroyed. When a constant amount of wheat and corn (0.5 gm.) was supplemented with increasing amounts of autoclaved yeast, there was at first a very

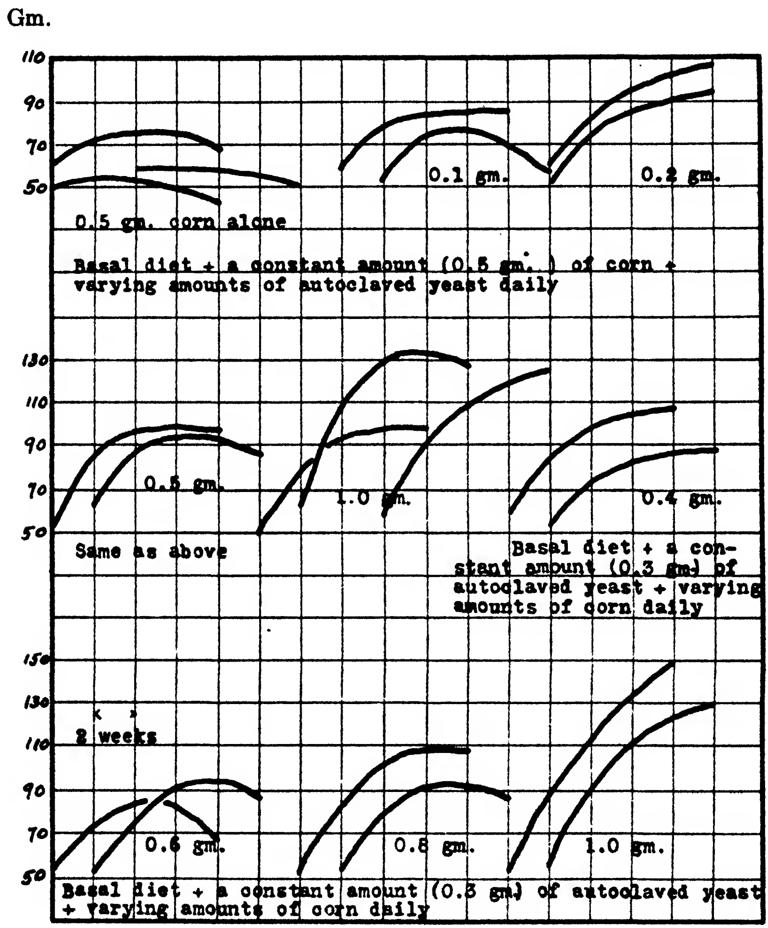

CHART 3.

noticeable stimulation in the growth of the rat, but loss in weight always followed. This can be accounted for, as due to the fact that the 0.5 gm. of the grains did not supply sufficient of the vitamin F for growth. The curves show that when a constant amount

of autoclaved yeast (0.3 gm.) was supplemented with varying amounts of wheat and corn, approximately 1.0 gm. of these grains was necessary to supply sufficient of the vitamin F for growth. This supplementary value of autoclaved yeast to wheat and corn

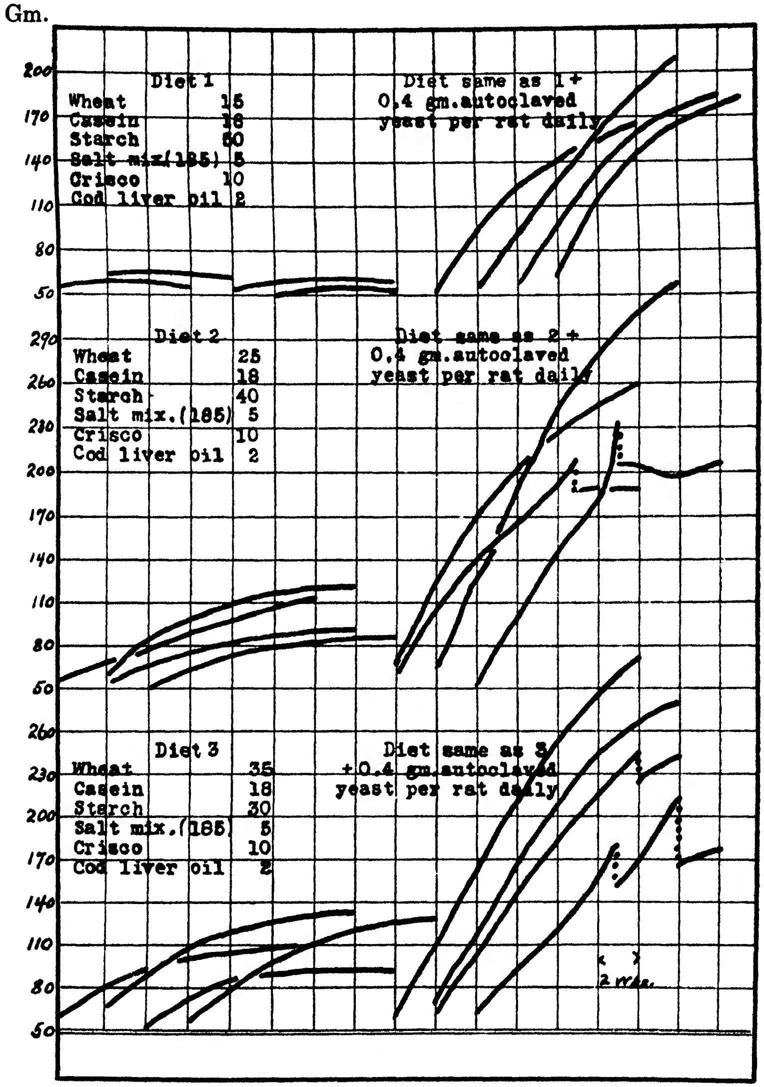

Chart 4.

shows that these grains are relatively rich in vitamin F, while the autoclaved yeast is rich in the growth-promoting factor or vitamin G. At least 1.0 gm. of wheat and corn is necessary to supply sufficient vitamin F for growth.

Another series of experiments was undertaken and instead of feeding the wheat separately it was incorporated into an otherwise vitamin B-free diet in the amounts of 15, 25, and 35 per cent. In a third series diets with the same amounts of wheat were supplemented with 0.4 gm. of autoclaved yeast fed separately to each rat daily. The growth curves of these rats are shown in Chart 4. The curves again show rather clearly that wheat is low in vitamin G.

All of the rats on the diets that were not supplemented with autoclaved yeast developed symptoms indicative of pellagra. The autoclaved yeast prevented these symptoms and also greatly influenced the rate of growth. It will be noticed that 15 per cent of wheat supplies very near the minimum requirements of vitamin F for growth but not enough for reproduction, as no young were ever born to these rats, while 25 and 35 per cent supply sufficient for growth and reproduction.

The data show that wheat and corn contain approximately the same amount of vitamin F, and that these grains are low in vitamin G. When this last factor was supplied by autoclaved yeast, good growth was obtained. Yeast contains both the vitamins F and G, and possibly a third factor which is now being investigated. The vitamin G, as has been shown, is thermostable, while vitamin F is thermolabile. The vitamin B complex is therefore composed of at least two vitamins.

This work confirms that of Sherman and Axtmayer (10) and extends the data to include corn.

SUMMARY.

Rats on a vitamin B-free diet supplemented with 0.3 gm. to 2.0 gm. of autoclaved yeast died in 5 to 9 weeks with polyneuritis or emaciation. The autoclaving of the yeast destroyed vitamin F (antineuritic).

When the growth of rats, receiving a mixture of either wheat or corn and autoclaved yeast, is compared with the growth of other rats receiving wheat or corn alone, it is seen that the autoclaved

yeast supplements the value of wheat and corn in inducing growth. This difference in growth supports the view that in wheat and corn the limiting factor for growth is vitamin G. The limiting factor is, in this case, found in autoclaved yeast.

15 per cent of wheat in the diet furnishes about the minimum amount of vitamin F for growth, provided sufficient vitamin G is present.

Therefore the vitamin B complex is composed of at least two vitamins. One which prevents polyneuritis in rats (vitamin F) and a second factor (vitamin G) which prevents experimental pellagra and together with vitamin F induces fair growth in rats.

Wheat and corn contain approximately the same amount of vitamins F and G, but they are richer in vitamin F than in vitamin G.

BIBLIOGRAPHY.

1. McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916–17, xxviii, 211.
2. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.
3. Guest, A. E., Nelson, V. E., Parks, T. B., and Fulmer, E. I., *Am. J. Physiol.*, 1926, lxxvi, 339.
4. Bell, M., and Mendel, L. B., *Am. J. Physiol.*, 1922, lxii, 145.
5. Croll, H. M., and Mendel, L. B., *Am. J. Physiol.*, 1925, lxxiv, 674.
6. Steenbock, H., Sell, M. T., and Nelson, E. M., *J. Biol. Chem.*, 1923, lv, 399.
7. Hunt, C. H., *Ohio Agric. Exp. Station, Research Bull. 415*, 1927.
8. Goldberger, J., and Lillie, R. D., *Pub. Health Rep., U. S. P. H. S.*, 1926, xli, 1025.
9. Chick, H., and Roscoe, M. H., *Biochem. J.*, 1927, xxi, 698.
10. Sherman, H. C., and Axtmayer, J. H., *J. Biol. Chem.*, 1927, lxxv, 207.
11. Emmett, A. D., and Luros, G. O., *J. Biol. Chem.*, 1920, xliii, 265.
12. Funk, C., and Dubin, H. F., *Proc. Soc. Exp. Biol. and Med.*, 1921, xix, 15.
13. Levene, P. A., and Muhlfeld, M., *J. Biol. Chem.*, 1923, lvii, 341.
14. Kinnersley, H. W., and Peters, R. A., *Biochem. J.*, 1925, xix, 820.
15. Hauge, S. M., and Carrick, C. W., *J. Biol. Chem.*, 1926, lxix, 403.
16. Smith, M. I., and Hendrick, E. G., *Pub. Health Rep., U. S. P. H. S.*, 1926, xli, 201.
17. Goldberger, J., Wheeler, G. A., Lillie, R. D., and Rogers, L. M., *Pub. Health Rep., U. S. P. H. S.*, 1926, xli, 297.
18. Salmon, W. D., *J. Biol. Chem.*, 1927, lxxiii, 483.
19. Salmon, W. D., Guerrant, N. B., and Hays, I. M., *J. Biol. Chem.*, 1928, lxxvi, 487.
20. Sherman, H. C., and MacArthur, E. H., *J. Biol. Chem.*, 1927, lxxiv, 107.

A GENERAL REACTION OF AMINO ACIDS.

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In the course of some experiments relative to the great variation shown by the lability of sulfur in cystine and its derivatives, it was desirable to prepare diacetylcystine, a substance which has not hitherto been described. Since the usual reagents gave indifferent results it was decided to try a mixture of acetic anhydride and pyridine. Upon warming the mixture on a steam bath, the cystine went into solution in the course of 2 or 3 hours and on removing the excess of reagents it was expected that the residue, which did not crystallize, would be mainly diacetylcystine. On dissolving the compound in boiling water, in addition to sulfur and hydrogen sulfide the formation of a substance reacting with phenylhydrazine was observed. It was suspected that the latter substance would prove to be pyruvic acid, the formation of which from cystine was long ago observed by Dewar and Gamgee (1). Examination of the phenylhydrazone quickly disproved this idea for not only were the physical properties of the substance, including the melting point of about 120°, widely different from pyruvic acid phenylhydrazone but in addition it contained much sulfur. Investigation showed that the hydrazone was mainly a derivative of a neutral ketone approximating in composition the formula $C_6H_{10}O_2NS$.

The formation of this product seemed sufficiently unusual to warrant further investigation and it was soon found that an analogous reaction took place when most amino acids were acted upon by a mixture of acetic anhydride and pyridine. The ketones derived from tyrosine and phenylalanine were particularly well characterized crystalline products while those obtained from other amino acids including leucine, β-trimethylalanine, glycine, alanine, histidine, and glutamic acid were readily converted into crystalline

derivatives. In the case of proline little or no ketone formation was observed. In every case the evolution of carbon dioxide was noted to accompany ketone formation. An investigation of the structure of the compounds indicated that two $CH_3{\cdot}CO$ groups were present, one being attached to nitrogen in the usual fashion and one attached to carbon in the form of a ketone. The reaction with phenylalanine may be represented as follows:

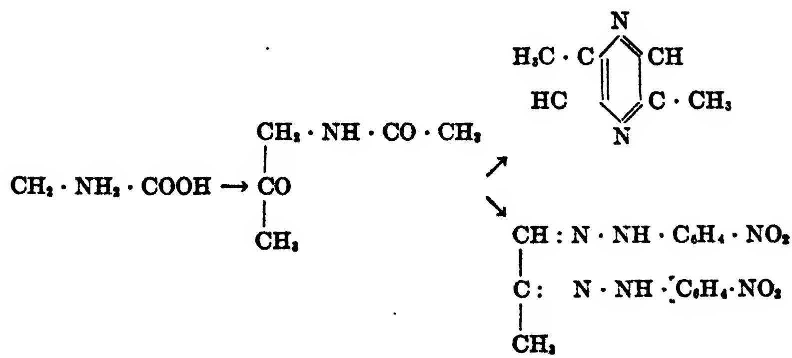
$$C_6H_5 \cdot CH_2 \cdot CHNH_2 \cdot COOH \rightarrow CO_2 + C_6H_5 \cdot CH_2 \cdot CH\begin{array}{l} \diagup CO \cdot CH_3 \\ \diagdown NH \cdot CO \cdot CH_3 \end{array}$$

It will be noted that the product represents a substituted acetylaminoacetone and the properties of the substance and those of its homologues are in entire accord with this supposition.

It will be convenient to consider first the evidence upon which the structure of these compounds is based and then to consider the possible mechanism of their formation.

(*a*) In the case of amino acids such as tyrosine, phenylalanine, and leucine, in which the reaction with acetic anhydride and pyridine proceeds to virtual completion, it was possible to show that almost exactly 1 molecule of carbon dioxide was evolved from each molecule of amino acid. The estimations were made by aspirating carbon dioxide-free air through the reacting mixture heated on a steam bath. The gases evolved were washed through a tube containing a little borax solution to remove acetic acid or pyridine and then absorbed in barium hydroxide of known strength. The carbon dioxide was determined by titration, phenolphthalein being used in the usual fashion. The yield of carbon dioxide varied from 85 to 97 per cent of the theoretical amount. (*b*) The presence of one acetyl group attached to nitrogen was indicated by hydrolysis with boiling 10 per cent sodium hydroxide, followed by steam distillation after acidification with phosphoric acid. (*c*) The presence of a ketone group was shown by the ready formation of derivatives with phenylhydrazine, semicarbazide, aminoguanidine, and similar bases. (*d*) Evidence that the ketone was a methylketone was obtained by noting the strong positive iodoform and nitroprusside reactions together with the formation of benzylidene derivatives on condensation with benzaldehyde in the presence of

dilute sodium hydroxide. (*e*) The position of the methylketone group was indicated by the fact that glycine, β-trimethylalanine, and phenylaminoacetic acid underwent the reaction in question and in these substances the α-carbon atom of the amino acid is the only possible place of attachment. (*f*) The representation of the products as derivatives of acetylaminoacetone was shown to be correct by the observation that after removal of the acetyl group attached to nitrogen by hydrolysis with acid, by the action of alkali, preferably in the presence of a mild oxidizing agent such as mercuric chloride, they are converted into pyrazine derivatives, just as aminoacetone was shown by Gabriel and Pinkus (2) to give 2,5-dimethylpyrazine. Thus the derivative from alanine gave tetramethylpyrazine. Also like aminoacetone they may be condensed with thiocyanic acid in acid solution but these compounds await further examination. (*g*) Lastly by the action of *p*-nitrophenylhydrazine in hot dilute sulfuric acid solution, the new ketones are corrected into the highly characteristic *bis*-nitrophenylhydrazones of glyoxals, just as aminoacetone gives methylglyoxal *bis*-nitrophenylhydrazone. The two last reactions (*f*) and (*g*) may be represented as follows in the case of the product from glycine:



Assuming that the foregoing evidence adequately justifies the structure, $R \cdot CH \cdot (CO \cdot CH_3) \cdot NH \cdot CO \cdot CH_3$, assigned to these derivatives, the next question is how to account for their formation for it will be generally conceded that the substitution of hydrogen attached to carbon in an aliphatic chain by a $CH_3 \cdot CO$ group is sufficiently unusual to warrant further consideration. At first the

possibility was considered that a diacetyl derivative of the amino acid was initially formed and that as the result of intramolecular rearrangement one acetyl group migrated from nitrogen to carbon. The resulting product would be a β-ketonic acid, which would be expected to lose carbon dioxide, giving the products in question:

$$R \cdot CHNH_2 \cdot COOH \rightarrow R \cdot CHN(COCH_3)_2 \cdot COOH \rightarrow$$

$$R \cdot C(CO \cdot CH_3) \cdot (NH \cdot COCH_3) \cdot COOH \rightarrow$$

$$R \cdot CH \cdot (COCH_3) \cdot NH \cdot CO \cdot CH_3 + CO_2$$

Such a change involving the migration of a $CH_3 \cdot CO$ group from nitrogen to carbon while sufficiently unusual would bear a certain resemblance to the formation of acylaminoketones from diacylanilides as observed by Chattaway (3). The likelihood of the preceding hypothesis was reduced almost to the vanishing point by the following observations. In the first place no similar reaction involving ketone formation takes place when a base such as benzylamine or β-phenylethylamine is heated with acetic anhydride and pyridine, although conditions should favor the initial production of diacetylated bases. Furthermore the reaction involving ketone formation from amino acids is to a certain extent specific to pyridine and its homologues including lutidine and collidine, while other bases such as dimethylaniline and quinoline, while certainly not interfering with acetylation do not bring about the formation of significant amounts of ketones, at least at temperatures which are effective with pyridine, namely about 80–90°. An attempt to settle the question whether diacetylation of the amino group was a necessary preliminary to ketone formation was made by using *N*-methyl- and *N*-dimethylaminophenylacetic acids. Obviously in the case of these substances diacetylation could not take place. When heated with acetic anhydride and pyridine carbon dioxide was not evolved and the final products of the reaction appeared to be almost entirely the monoacetyl derivative. Sarcosine gave similar results and though a little carbon dioxide was evolved no ketone could be identified in either case. A much more definite answer to the problem in question was obtained by the observation that the reaction with acetic anhydride and pyridine was not restricted to amino acids and that an analogous reaction—though often complicated by side

changes—took place with acids containing no nitrogen and incapable of acetylation. Thus while hydroxy acids appeared not to react, acids as different as chloroacetic, phenylbromoacetic, and α-bromostearic acids, all underwent at varying speeds the reaction typical of the amino acids with evolution of carbon dioxide. Hippuric acid behaved like glycine, the benzyl group being replaced by acetyl. Even an unsubstituted acid such as phenylacetic acid on being heated with pyridine and acetic anhydride was converted into methylbenzylketone. The change may be represented as follows:

$$C_6H_5 \cdot CH_2 \cdot COOH \rightarrow C_6H_5 \cdot CH \cdot (CO \cdot CH_3) \cdot COOH \rightarrow C_6H_5 \cdot CH_2 \cdot CO \cdot CH_3$$

In the light of the foregoing evidence it may be taken as certain that migration of an acetyl group from nitrogen to carbon is not involved in the formation of these ketones from amino acids and that diacetylation if it occurs at least is not essential.

On the other hand the reaction bears a close resemblance to certain changes observed over 40 years ago by W. H. Perkin, senior (4). Acetone for example resulted from heating at 180–200° acetic anhydride with sodium acetate in sealed tubes, and proprione and butyrone were similarly obtained. Perkin interpreted the action along lines analogous to those adopted by him to represent his cinnamic acid synthesis but Fittig (5) 10 years later without further experimental data indicated the probability that β-ketonic acids were first formed and then decomposed to the ketone and carbon dioxide.

$$\left.\begin{array}{l} R \cdot CH_2 \cdot CO \\ R \cdot CH_2 \cdot CO \end{array}\right\rangle O + CH_3COOH \rightarrow R \cdot CH_2 \cdot CO \cdot CH_2 \cdot COOH + R \cdot CH_2 \cdot COOH$$

$$R \cdot CH_2 \cdot CO \cdot CH_2 \cdot COOH \rightarrow R \cdot CH_2 \cdot CO \cdot CH_3 + CO_2$$

So far as we are aware little or no attention has been directed during recent years to these early experiments of Perkin.

The accomplishment of a similar reaction to that of Perkin at relatively low temperatures, with amino acids as one component and pyridine as a kind of catalyst, is somewhat surprising. Assuming that the analogy between Perkin's reaction and the change under discussion is well founded, the sequence of events leading to

an acetylated aminoketone from phenylalanine may be represented thus:[1]

$$C_6H_5 \cdot CH_2 \cdot CHNH_2 \cdot COOH \rightarrow C_6H_5 \cdot CH_2 \cdot CHNH(CO \cdot CH_3) \cdot COOH \rightarrow$$
$$C_6H_5 \cdot CH_2 \cdot C(CO \cdot CH_3) \cdot NH(CO \cdot CH_3) \cdot COOH \rightarrow$$
$$C_6H_5 \cdot CH_2 \cdot CH(COCH_3)NH \cdot CO \cdot CH_3 + CO_2$$

It may be noted that in conformity with the above scheme it was found that α-aminohydratropic acid, $C_6H_5 \cdot C(NH_2)CH_3 \cdot COOH$, which contains no unsubstituted hydrogen atom in the α position, failed to undergo the reaction typical of the ordinary α-amino acids. The homologues of acetic anhydride are much less reactive than the latter substance. Acid chlorides such as acetyl chloride react vigorously but less smoothly than the anhydrides.

Reference may be made at this point to a paper that recently appeared by Levene and Steiger (6) on the action of acetic anhydride, acetone, and pyridine on tyrosine and phenylaminoacetic acid. Two of the tyrosine derivatives described by these authors have the same elementary composition as those described in the present communication but the product $C_{14}H_{17}O_4N$ differs in melting point by some 30°. Structural formulæ were suggested by Levene and Steiger for these compounds containing isopropylidene groups which, for reasons that do not seem clear, were supposed to arise from acetone derived from or present in the acetic anhydride or deliberately added. The possible identity of Levene and Steiger's compounds with the ketones from tyrosine described by us remains open. The properties of our tyrosine derivatives are certainly not those indicated by the provisional formulæ suggested by Levene and Steiger, although the ketone from phenylaminoacetic acid is probably identical with their product and has one of the alternative formulæ proposed by them.

[1] It will be recalled that Victor Meyer (*Ber. chem. Ges.*, 1893, xxvi, 1365) showed that in certain cases the (OH) group in the carboxyl group of amino acids was capable of acetylation with formation of what is virtually a mixed anhydride. Thus p-dimethylaminobenzoic acid gave

$$C_6H_4 \left\langle \begin{array}{l} N(CH_3)_2 \\ CO \cdot O \cdot CO \cdot CH_3 \end{array} \right.$$

It is possible that an analogous reaction represents a step in the change under discussion but it is certainly not a necessary assumption with the evidence at present available.

It may be observed that the acetylaminoketones described in this paper contain an asymmetric carbon atom, but when made from optically active amino acids, they were invariably inactive. Their mode of formation would lead one to suppose that racemization might readily occur.

A word may be said as to possible biological analogies with the reaction with which we are concerned. The synthesis of β-ketonic acids—*i.e.* the reverse of β-oxidation—at reasonably low temperatures, through the catalytic action of a base, is suggestive and will be further explored. The observation was made that the catalytic action of pyridine in this reaction could also be exercised by nicotinic acid—pyridine-β-carboxylic acid—a substance closely associated with Funk's vitamin preparations from rice polishings and yeast. Possibly this association is purely fortuitous and in any case we refrain from further speculation at this time. A reaction involving the removal of carbon dioxide from α-amino acids with the formation of amine derivatives which in some cases are related to the pressor amines, is also significant and should lead to the preparation of pharmacologically interesting substances. As was to be expected the acetylated aminoketone derived from tyrosine showed little or no pressor effect but on reduction with sodium and alcohol a derivative was obtained, presumably $C_6H_4OH \cdot CH_2 \cdot CH \cdot (CHOH \cdot CH_3) \cdot NH_2$, which showed some activity. Some histidine derivatives were much more active and will be studied further. We are indebted to Dr. Lieb for making these preliminary pharmacological tests. Lastly the ready conversion of amino acids into pyrazine derivatives, some of which are found in fusel oil, is noteworthy.

EXPERIMENTAL.

Phenylalanine.—This amino acid was converted into benzylacetylaminoacetone as follows: The synthetic acid (3 gm.) was heated on a steam bath with pyridine (10 cc.) and acetic anhydride (15 cc.) for 5 hours. Carbon dioxide was evolved freely after about 10 minutes and most of the reaction was over in an hour. The mixture was distilled in steam until about 150 cc. of distillate had collected. Toward the end of the distillation an oil begins to separate out from the residue. The mixture was cooled, neutralized with sodium bicarbonate, and extracted four times with

ether. On evaporation of the ether the residue which weighed 3.1 gm. readily solidified in the form of clumps of waxy prisms. The crude product when dried on tile melted at 96°. It is very soluble in alcohol, acetone, and ethyl acetate, sparingly soluble in water. It is best crystallized from xylene, separating in clusters of long thin needles melting sharply at 98–99°. An aqueous solution gives an immediate precipitate of iodoform on adding iodine and sodium hydroxide. With sodium nitroprusside and sodium hydroxide an intense dark cherry-red color is produced which assumes a slightly violet tinge on acidifying with acetic acid. Ammoniacal silver nitrate, Fehling's solution, and mercuric salts in the presence of alkali are all reduced on boiling. On heating with zinc dust it gives a strong pyrrole reaction. Small amounts may be distilled unchanged in a high vacuum.

Analysis. $C_{12}H_{15}O_2N$.

Calculated.	C 70.2,	H 7.32,	N 6.83.
Found.	" 70.5,	" 7.18,	" 6.80.

The *semicarbazone* was prepared by dissolving 0.5 gm. of the ketone in a little alcohol and adding semicarbazide hydrochloride (0.3 gm.) and potassium acetate (0.3 gm.) dissolved in water. The semicarbazone separates out as an oil at first and is rather hard to crystallize. It crystallizes from alcohol in needles melting at 103–104°.

Analysis. $C_{13}H_{18}O_2N_4$.

Calculated.	C 59.5,	H 6.87.
Found.	" 59.7,	" 6.95.

The *phenylhydrazone* was prepared by dissolving the ketone in a little 50 per cent acetic acid and adding a solution of phenylhydrazine in dilute acetic acid. An oily hydrazone separates at once. It is sparingly soluble in hot water, readily soluble in alcohol. It was not analyzed. The *aminoguanidine derivative* crystallizes from water in stout prisms in which it is sparingly soluble and melts at 180–181° with evolution of gas.

Tyrosine.—The optically active amino acid was used. The product, namely *p*-hydroxybenzyl-acetylaminoacetone was readily obtained as follows: Tyrosine (6 gm.) was heated on the steam bath with pyridine (20 cc.) and acetic anhydride (20 cc.) for about 6 hours. The tyrosine dissolves slowly and the evolution of carbon

dioxide was noticeable after half an hour or less. The bulk of the acetic acid and pyridine was removed by a short steam distillation. The residue contains the O-acetyl derivative of the desired product and this may be crystallized out directly by concentrating the solution. It is very soluble in water and in most organic solvents. It crystallizes best from dilute acetic acid on slow evaporation and melts at 123° with some slight softening.

Analysis. $C_{14}H_{17}O_4N$.

Calculated.	C 63.8,	H 6.51, N 5.32.
Found.	" 63.7,	" 6.70, " 5.35.

In order to obtain the free hydroxybenzyl-acetylaminoacetone the separation of the preceding compound is neither necessary nor desirable. A moderate excess of sodium carbonate is added to the contents of the distillation flask and steam is blown through for about 20 minutes. In this way the acetyl group attached to the phenolic hydroxyl is removed and at the same time residual pyridine is got rid of. The solution is filtered, if necessary, to remove a trace of oily matter and then extracted four times by shaking with butyl alcohol. The ketone is readily extracted and on removal of the solvent it crystallizes easily. It is best recrystallized from water with a little charcoal, and separates in the form of stout prisms which melt at 135–136°. The yield is almost equal to that of the tyrosine from which it is prepared. The substance even when pure frequently separates out as an oil which readily solidifies on standing. It is sparingly soluble in ether or chloroform, readily soluble in alcohol and ethyl acetate. Its aqueous solution gives a neutral reaction to litmus, and gives the iodoform and cherry-red nitroprusside reactions. It gives a strong pyrrole reaction on distillation with zinc dust. It yields no amino nitrogen on treatment with nitrous acid.

Analysis. $C_{12}H_{15}O_3N$.

Calculated.	C 65.1,	H 6.84, N 6.33.
Found.	" 65.0,	" 6.80, " 6.20.

The *molecular weight* was determined by Barger's method. A $\frac{1}{8}$ N cane sugar solution was in equilibrium with a 1.90 per cent aqueous solution of the substance, indicating a molecular weight of 211 as against a theoretical value of 221.

An estimation of the acetyl group was made by boiling 0.5 gm. with 15 cc. of 10 per cent sodium hydroxide for 3 hours. On acidifying with phosphoric acid and distilling the filtrate with steam, acetic acid equivalent to 20.6 per cent was obtained, compared with a calculated value of 19.5.

The *semicarbazone* was prepared by warming an aqueous 10 per cent solution of the substance with semicarbazide (1 mol) and sodium bicarbonate (1 mol). Crystals promptly separate which are very sparingly soluble even in boiling water. The yield is quantitative. On recrystallization from water, stout cubic prisms are obtained melting at 230–231° with evolution of gas.

Analysis. $C_{13}H_{18}O_3N_4$.

Calculated.	C 56.1,	H 6.48.
Found.	" 56.4,	" 6.95.

A beautifully crystalline *picrate* is obtained by dissolving 0.5 gm. of *p*-hydroxybenzyl-acetylaminoacetone in hot water and adding picric acid (0.6 gm.) also dissolved in hot water. The picrate separates in the form of large thin golden plates with a curious silvery luster. It is readily soluble in hot water and moderately so in cold. The recrystallized substance melts at 125–126°.

The *phenylhydrazone* is best prepared by adding phenylhydrazine dissolved in dilute acetic acid to a cold aqueous solution of the ketone. It is precipitated as a compact white powder which turns slightly yellow on long exposure. It is very soluble in alcohol, moderately soluble in benzene, and insoluble in petroleum ether, and is difficult to crystallize. The crude substance melts in the neighborhood of 100°. On dissolving the hydrazone in warm 10 per cent hydrochloric acid, a bulky mass of fine needles of the hydrochloride separates on cooling. The hydrochloride melts at 195–197° with evolution of gas with some previous sintering.

Analysis. $C_{18}H_{21}O_2N_3$.

Calculated.	C 69.4,	H 6.75,	N 13.5.
Found.	" 69.6,	" 6.90,	" 13.4.

The *benzylidene* derivative of *p*-hydroxybenzyl-acetylaminoacetone was prepared as follows: The ketone (1 gm.) was dissolved in 10 cc. of 0.5 N sodium hydroxide. Benzaldehyde (0.6 gm.)

together with a little alcohol was then added and after shaking allowed to stand overnight. A trace of unchanged aldehyde was removed by filtration and carbon dioxide was then passed into the filtrate. The benzylidene derivative separated at once as a white precipitate very sparingly soluble in water, very soluble in alcohol and acetic acid. It is difficult to recrystallize from organic solvents but may be purified by solution in a little sodium hydroxide and reprecipitation with carbon dioxide. The yield is about 80 per cent of the theoretical amount. The substance has no sharp melting point but begins to decompose above 150° but does not melt completely until between 170–180°.

The positive catalytic action of other substances such as isoquinoline, lutidine, collidine, and nicotinic acid was established by heating them with acetic anhydride and tyrosine exactly as described in the case of pyridine, noting the evolution of carbon dioxide, and isolating the ketone. The failure of quinoline and dimethylaniline to induce the reaction was shown by absence of carbon dioxide evolution and the formation of no ketones.

Phenylaminoacetic Acid.—The preparation of α-phenyl-α-acetaminoacetone from this amino acid was carried out under precisely the same conditions as those used for phenylalanine. The ketone is freely soluble in alcohol, less so in ether, and moderately soluble in water. It crystallizes in glistening plates melting at 103–104° and shows the usual ketone reactions of its homologues. The yield is almost the theoretical amount.

Analysis. $C_{11}H_{13}O_2N$.

Calculated.	C 69.1,	H 6.85,	N 7.33.
Found.	" 69.3,	" 6.97,	" 7.21.

On treating the substance even in the cold with sodium hydroxide, ammonia is slowly evolved owing to deep seated decomposition, while on warming on the steam bath with 5 parts of 20 per cent hydrochloric acid for an hour and then concentrating, hydrolysis proceeds normally and the hydrochloride of α-phenyl-α-aminoacetone is obtained in excellent yield. This substance has already been obtained by Kolb (7) and the products agree in every particular. It turns red at about 200° and melts at 204–208° according to the rate of heating. On treating the hydrochloride with sodium hydroxide it is rapidly converted into 3,6-dimethyl-2,5-diphenylpyrazine.

Leucine.—This amino acid was readily converted into α-isobutyl-α-acetaminoacetone. 3 gm. were warmed on the steam bath with pyridine (10 cc.) and acetic anhydride (10 cc.) for 6 hours. On steam distillation a trace of ketone was found in the distillate but almost all remained in solution in the residue. This was saturated with sodium bicarbonate and extracted four times with ether. On evaporation of the ether solution the ketone (3.0 gm.) remains as a clear slightly yellow oil which did not crystallize even in a freezing mixture or after long keeping. It is fairly soluble in water and freely soluble in most organic solvents except petroleum. It gives intense iodoform and nitroprusside reactions. The substance was dried *in vacuo* for analysis.

Analysis. $C_9H_{17}O_2N$.

Calculated.	C 63.2, H 9.94, N 8.18.
Found.	" 62.9, " 10.20, " 8.20.

The *aminoguanidine* derivative is the most attractive derivative and is readily obtained by warming an aqueous solution of the ketone and aminoguanidine in equivalent amounts. It crystallizes readily from boiling water in the form of stout prisms melting at 182–183°. It is sparingly soluble in cold water.

Analysis. $C_{10}H_{21}ON_5$.

Calculated.	C 52.9, H 9.25.
Found.	" 52.9, " 9.37.

The *semicarbazone* prepared in the usual way, separates as an oil which slowly solidifies. It is very soluble in water and alcohol and moderately soluble in chloroform. It is best crystallized by dissolving in a little absolute alcohol and adding petroleum ether, when it separates as well formed prisms. The substance does not melt sharply but undergoes some change around 170°, again turns solid, and finally melts at 215–218°. The *4,4-diphenylsemicarbazone* has similar properties and does not crystallize more readily.

The *phenylhydrazone* is obtained as a slowly solidifying oil when aqueous solutions are used but is better prepared by mixing equivalent amounts of the ketone and phenylhydrazine in a little dry ether. It forms glistening almost colorless prisms, slowly turning yellow on exposure, and melting at 110–113°.

Alanine.—The amino acid (4 gm.) was heated with pyridine (10 cc.) and acetic anhydride (25 cc.). Carbon dioxide was

evolved steadily but the reaction was slower than with the higher homologues. Heating was continued for 6 hours and then the mixture was distilled with steam. Only a trace of ketone was in the distillate. A slight excess of sodium carbonate was then added and a little residual pyridine removed by a further short steam distillation. The residue was then extracted with butyl alcohol repeated five times. On removing the solvent 3 gm. of clear colorless oil were obtained which did not crystallize. It gave all the ketone reactions in typical fashion. The most convenient crystalline derivative was that obtained from aminoguanidine which crystallizes in stout prisms melting at 183–184°. It is readily soluble in hot water, sparingly soluble in cold.

Analysis. $C_7H_{15}ON_5$.

Calculated.	C 45.4, H 8.11.
Found.	" 45.7, " 8.23.

The constitution of the alanine derivative was proved to be α-methyl-α-acetaminoacetone by two very convincing reactions. In the first place on boiling the ketone with phenylhydrazine in 2 per cent sulfuric acid, the very characteristic *bis*-phenylhydrazone of diacetyl quickly separated out. It melted at 240°, and was identical with the product previously obtained by Pechmann (8) and others.

Secondly, on heating the ketone for an hour with 5 parts of 20 per cent hydrochloric acid and then concentrating, a syrupy residue of the hydrochloride of α-methyl-α-aminoacetone was obtained. On distilling this residue with potash and mercuric chloride according to the conditions described by Künne (9) an excellent yield of the highly characteristic crystalline hydrate of tetramethylpyrazine was obtained, melting at 74–75°.

Glycine and Hippuric Acid.—The reaction of glycine with acetic anhydride and pyridine does not proceed as smoothly as with the other amino acids. The yield of carbon dioxide under the conditions used for other amino acids was only about 35 to 40 per cent of the theoretical amount and considerable darkening of the mixture takes place on heating. On steam distillation little or no ketone was in the distillate, while the residue showed all the typical reactions. It was not found possible to isolate the pure acetaminoacetone but its presence was established by using the

two reactions previously described in the case of the ketone from alanine. In the first place the aqueous solution was boiled with excess of *p*-nitrophenylhydrazine in 5 per cent sulfuric acid. For a few moments no obvious reaction occurs and then the separation of the characteristic scarlet *bis*-*p*-nitrophenylhydrazone of methylglyoxal begins. The yield of product amounts to about half the weight of glycine used. It was crystallized from nitrobenzene and melted at 300–302°, and gave the characteristic blue color with alcoholic sodium hydroxide (10). It contained 24.5 per cent of nitrogen, compared with a calculated value of 24.6.

Confirmatory evidence of the presence of acetaminoacetone was obtained by acid hydrolysis, followed by distillation with mercuric chloride and alkali as described for the ketone from alanine. A good yield of 2,5-dimethylpyrazine was obtained which was identified by both the gold salt m.p. 152–153° and picrate m.p. 157°.

The behavior of hippuric acid when heated with acetic anhydride and pyridine exactly paralleled that of glycine with the single difference that steam distillation revealed the presence of much benzoic acid, resulting from the replacement of the benzoyl group by acetyl. The aqueous solution after steam distillation was treated as just described and gave the methylglyoxal and 2,5-dimethylpyrazine derivatives.

The behavior of other amino acids toward acetic anhydride and pyridine is the subject of further investigation.

SUMMARY.

A reaction which appears general to α-amino acids is described. On warming amino acids with acetic anhydride and pyridine, carbon dioxide is evolved and two acetyl groups are introduced, one attached to nitrogen and one to carbon. The compounds have the general formula $R \cdot CH \cdot (NH \cdot COCH_3) \cdot CO \cdot CH_3$ and are derivatives of acetylaminoacetone.

Proof of the constitution of the products is adduced and various derivatives described as well as their conversion into glyoxal and pyrazine derivatives.

Proline and alkylamino acids do not react analogously but undergo simple acetylation. The same is true of α-aminohy-

dratropic acid which contains no unsubstituted hydrogen in the α position.

The reaction between α-amino acids, pyridine, and acetic anhydride has certain analogies to a change observed long ago by W. H. Perkin, senior, who obtained acetone by heating acetic anhydride and sodium acetate at high temperatures. It is believed that β-ketonic acids represent intermediate stages of the reaction, ketones being formed by loss of carbon dioxide.

The function of the pyridine in the reaction appears to be catalytic and is not shared by dimethylaniline or quinoline. On the other hand pyridine derivatives such as lutidine, collidine, and nicotinic acid were effective.

Reference is made to the fact that the reaction is not limited to amino acids but is shared by α-halogen acids and some unsubstituted acids such as phenylacetic acid which gives methylbenzylketone. The possible biological significance of the reaction is discussed together with reference to the possible uses of alkyl acetaminoacetones for the preparation of pharmacologically active substances.

BIBLIOGRAPHY.

1. Dewar, J., and Gamgee, A., *J. Anat. and Physiol.*, 1870, vii, 142.
2. Gabriel, S., and Pinkus, G., *Ber. chem. Ges.*, 1893, xxvi, 2797.
3. Chattaway, F. D., *J. Chem. Soc.*, 1904, lxxxv, 386.
4. Perkin, W. H., *J. Chem. Soc.*, 1886, xlix, 317.
5. Fittig, R., *Ber. chem. Ges.*, 1897, xxx, 2150.
6. Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, 1927, lxxiv, 689.
7. Kolb, A., *Ann. Chem.*, 1896, ccxci, 276.
8. Pechmann, H., *Ber. chem. Ges.*, 1887, xx, 3164.
9. Künne, H., *Ber. chem. Ges.*, 1895, xxviii, 2040.
10. Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, 1913, xv, 132.

THE DISTRIBUTION OF SUGAR BETWEEN BLOOD CORPUSCLES AND BLOOD PLASMA FOR SEVERAL ANIMAL SPECIES.*

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A number of investigations to determine the distribution of sugar between blood corpuscles and blood plasma have been performed on human blood. Wu (1), in reviewing this literature and adding to it some of his own analyses, concluded that the blood sugar is about equally divided between blood corpuscles and blood plasma. Wishart (2), in addition to working with human blood, did some work on dog, pony, goat, and sheep blood. She did not determine the per cent of the total sugar that was contained in the blood corpuscles but, by use of her published results as to sugar in corpuscles and plasma together with the corpuscle volumes given in her data, the following averages may be obtained. In dog blood an average of 20.2 per cent of the total blood sugar was in the corpuscles, in pony blood the average was 74.5 per cent, in goat blood it was 12.3 per cent, and in sheep blood it was 47.7 per cent. These figures would indicate that there is a marked species variation as to the distribution of the blood sugar between corpuscles and plasma.

Since work involving the determination of corpuscle and plasma sugar for several additional species was being conducted, it seemed of interest to collect these analyses with the view of noting species differences in distribution.

* Part of the work reported in this paper was done during the summer (1927) at the Iowa Lutheran Hospital, Des Moines, Iowa. I take pleasure in expressing my gratitude to Dr. Harry W. Dahl for the privileges of his laboratory.

TABLE I.

Animal No.	Sugar in corpuscles.	Sugar in plasma.	Whole blood sugar (calculated).	Corpuscle volume.	Per cent of total sugar in corpuscles.
Swine.					
	mg. per cent	*mg. per cent*	*mg. per cent*	*per cent*	
380 A	32	125	80	52	19
389 B	46	85	65	51	35
380 B	33	129	88	48	16
388 A	40	95	71	43	24
389 A	39	112	77	48	24
Average...	38	109	76	48	24
Guinea pigs.					
1	132	127	130	56	57
2	152	143	148	52	53
3	166	163	164	35	35
4	136	125	130	49	52
5	111	128	120	48	44
Average...	139	137	138	48	48
Cattle.					
J 114	29	43	38	41	31
H 55	31	58	46	46	32
5060	29	59	46	45	29
1414	32	60	48	40	27
Average...	30	55	45	43	30
Rabbits.					
60	31	83	58	48	25
44	30	127	88	40	14
45	29	102	65	50	22
49	62	114	98	31	19
62	31	129	94	36	12
56	38	141	98	42	16
51	38	107	69	56	31
Average...	37	115	81	43	20

TABLE I.—*Concluded.*

Animal No.	Sugar in corpuscles.	Sugar in plasma.	Whole blood sugar (calculated).	Corpuscle volume.	Per cent of total sugar in corpuscles.
		Human.			
V. O.	120	104	111	45	48
E. H.	117	115	116	44	45
M. S.	133	116	124	45	48
F. M.	132	105	115	37	42
U. H.	103	91	97	48	51
M. C.	121	96	107	46	52
F. L.	121	91	105	49	56
Average...	121	103	111	45	49

EXPERIMENTAL.

Sugar determinations on the blood of cattle, swine, rabbits, guinea pigs, and human beings were carried out. The sugar in the blood from humans and guinea pigs was determined by Myers and Bailey's modification of the Lewis-Benedict method (3) with potassium oxalate as the anticoagulant, and that of cattle, swine, and rabbits by the new Folin method (4) with sodium citrate, 2 mg. per cc., as the anticoagulant. Sodium citrate in this amount was found not to alter significantly the results with the new Folin method. While the first method gave much higher values than the second, it was found that the per cent of the total blood sugar in the corpuscles was the same for similar blood samples irrespective of the method employed. The amounts of sugar in corpuscles and plasma were determined directly and the whole blood sugar was calculated. With the exception of those for cattle and swine, the values given represent fasting blood sugars. Blood from the cattle and the swine was drawn between 4 and 5 hours after feeding. The results are given in Table I.

DISCUSSION AND SUMMARY.

The data presented, although limited as to the number of individuals in each species, indicate that the distribution of sugar between blood corpuscles and blood plasma varies with species. That this variation is not dependent upon corpuscle

volume is demonstrated by the relatively constant values obtained for the various species as compared with the values for per cent of total sugar in corpuscles. No explanation for the species difference in sugar distribution is apparent. A possible explanation is suggested by some work of Glassman's (5) in which both free and combined glucose were determined. His data indicate that there is no free glucose in the corpuscles after a triple washing in physiological salt solution. If it is a fact that corpuscles contain no free glucose, then the variations found as regards species may indicate that there is a species difference in the stability of the glucose-protein compound in corpuscles and hence a difference in the degree of hydrolysis resulting when an acid protein precipitant is employed as was the case in the work here reported.

BIBLIOGRAPHY.

1. Wu, H., *J. Biol. Chem.*, 1922, li, 21.
2. Wishart, M. B., *J. Biol. Chem.*, 1920, xliv, 563.
3. Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.
4. Folin, O., *J. Biol. Chem.*, 1926, lxvii, 357.
5. Glassman, B., *Z. physiol. Chem.*, 1925, cl, 16; 1926, clviii, 113.

THE DISTRIBUTION OF SUGAR BETWEEN CORPUSCLES AND PLASMA IN BLOOD FROM NORMAL HUMAN BEINGS, AND FROM DIABETICS WITH AND WITHOUT INSULIN THERAPY.*

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INTRODUCTION.

In a condition characterized by a marked quantitative disturbance in blood sugar as is diabetes mellitus, it would not seem impossible that there might also be a disturbance in the distribution of sugar between corpuscles and plasma. Wu (1), in reviewing the literature and adding to it some of his own analyses, concluded that the blood sugar normally is about equally divided between blood corpuscles and blood plasma in human blood. That this ratio need not be a constant is indicated by a variable distribution of sugar between corpuscles and plasma in blood from different animal species (2). Secker's (3) *in vitro* experiments with insulin suggest that an abnormal distribution of sugar between corpuscles and plasma might be found in diabetes.

However, Wishart (4), in experimental diabetes in dogs, found no marked alteration in the distribution of sugar between corpuscles and plasma, and the distribution observed by Allen, Stillman, and Fitz (5) in the blood of human diabetics under dietary management is not far from that given by Wu for blood from normal individuals.

* The work reported in this paper was done during the summer (1927) at the Iowa Lutheran Hospital, Des Moines, Iowa. I take pleasure in expressing my gratitude to Dr. Harry W. Dahl for the privileges of his laboratory, and to Dr. Edwin B. Winnett for his kindness in obtaining blood samples for me from patients in his practice.

The following data are published in confirmation of the work of Wishart, and of Allen, Stillman, and Fitz with additional observations concerning the effect of insulin therapy on the blood sugar distribution in diabetics.

EXPERIMENTAL.

Blood was obtained from ten normal individuals, ten diabetics who had never had insulin, and ten diabetics who at the time were on insulin therapy. For the most part it was drawn from the subject after an overnight fasting period. Several of the blood samples, however, were taken between 3 and 4 hours after the subject had eaten.

TABLE I.

Normal Individuals.

Patient No.	Corpuscle sugar.	Plasma sugar.	Whole blood sugar (calculated).	Corpuscle volume.	Per cent of total sugar in corpuscles.
	mg. per cent	*mg. per cent*	*mg. per cent*	*per cent*	
1. V. M.	132	101	116	47	54
2. S. E.	123	128	125	50	48
3. U. H.	103	91	97	48	51
4. M. Y.	121	96	107	46	52
5. F. O.	121	91	105	49	56
6. L. E.	163	90	130	55	69
7. M. D.	118	95	104	37	42
8. M. S.	133	116	124	45	48
9. H. B.	117	115	116	44	45
10. B. E.	147	122	130	34	39
Average	126	103	115	45	50

Corpuscle volume was obtained by centrifugalization in graduated tubes. Myers and Bailey's (6) modification of the Lewis-Benedict method was used in the sugar determinations. Sugar in corpuscles and plasma was determined directly and whole blood sugar calculated from these on the basis of corpuscle volume. In the values given in Tables I to III for per cent of total sugar in corpuscles, the corpuscle volume was used in making the calculations.

TABLE II.

Diabetics Who Have Never Received Insulin.

Patient No.	Corpuscle sugar.	Plasma sugar.	Whole blood sugar (calculated).	Corpuscle volume.	Per cent of total sugar in corpuscles.
	mg. per cent	*mg. per cent*	*mg. per cent*	*per cent*	
11. B. N.	155	128	144	58	62
12. M. K.	282	270	274	33	34
13. S. H.	170	182	177	43	41
14. F. Y.	154	141	148	53	55
15. A. R.	130	118	125	58	60
16. Y. I.	213	222	218	44	43
17. L. H.	476	500	486	58	56
18. L. E.	476	571	526	48	43
19. G. G.	206	217	212	49	47
20. C. N.	170	158	164	58	60
Average	243	251	247	50	50

TABLE III.

Diabetics Who Were Receiving Insulin.

Patient No.	Corpuscle sugar.	Plasma sugar.	Whole blood sugar (calculated).	Corpuscle volume.	Per cent of total sugar in corpuscles.
	mg. per cent	*mg. per cent*	*mg. per cent*	*per cent*	
21. H. H.	400	426	412	52	50
22. M. R.	385	392	389	35	34
23. S. O.	222	233	229	36	39
24. W. R.	426	444	435	48	47
25. L. V.	328	345	337	45	44
26. R. S.	308	244	273	46	51
27. J. S.	263	241	250	42	44
28. M. M.	291	286	287	36	36
29. S. N.	333	314	321	39	40
30. R. E.	168	143	154	45	49
Average	312	307	309	42	44

DISCUSSION.

In accordance with earlier work, no abnormality was found to exist in the distribution of sugar between corpuscles and plasma in diabetic blood. The average amount of sugar in the corpuscles of both normal individuals and diabetics not being treated with insulin was 50 per cent of the total blood sugar. For diabetics who were at the time receiving insulin daily the average amount of sugar in the corpuscles was 44 per cent of the total blood sugar. Mathematically, the values for per cent of sugar in corpuscles for diabetics receiving insulin were found not to vary significantly when compared with similar values for diabetics not receiving insulin therapy or normal individuals.

No correlation was found to exist between the amount of sugar in the corpuscles and the corpuscle volume. There was, however, a very marked correlation between the values for corpuscle sugar and plasma sugar in both of the diabetic series, indicating that there is a free exchange of sugar between corpuscles and plasma. No correlation between values for corpuscle and plasma sugar could be found in the series of normal individuals. This does not mean that in normal blood free exchange of sugar between corpuscles and plasma fails to take place but rather that in blood from a non-diabetic there are a number of factors at work tending to alter the amount of sugar present in either corpuscles or plasma, making a definite correlation unlikely, whereas in diabetic blood there is one factor at work tending to raise the blood sugar and if there is a free exchange of sugar between corpuscles and plasma a definite correlation would be expected.

SUMMARY AND CONCLUSIONS.

1. There is no abnormality in the distribution of sugar between blood corpuscles and blood plasma in diabetic individuals.

2. Insulin therapy causes no significant alteration in distribution of sugar between the blood corpuscles and blood plasma.

3. The correlation found to exist between blood corpuscle sugar and blood plasma sugar in diabetics indicates that there is a free exchange of sugar between corpuscles and plasma.

BIBLIOGRAPHY.

1. Wu, H., *J. Biol. Chem.*, 1922, li, 21.
2. Shope, R. E., 1928, lxxviii, 107.
3. Secker, J., *J. Physiol.*, 1925, lx, 286.
4. Wishart, M. B., *J. Biol. Chem.*, 1920, xliv, 563.
5. Allen, F. M., Stillman, E., and Fitz, R., Total dietary regulation in the treatment of diabetes, Monographs of The Rockefeller Institute for Medical Research, No. 11, New York, 1919.
6. Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.

THE DISTRIBUTION OF SUGAR IN NORMAL HUMAN BLOOD.

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In a previous paper (1) we reported that the non-fermentable substances of blood, which reduce alkaline copper solutions, are not evenly distributed between the formed elements and the plasma, the former containing about 5 times as much of "reducing non-sugars" as the latter. From this it follows that the ratio, $\frac{\text{corpuscle sugar}}{\text{plasma sugar}}$, derived from apparent sugar (total reduction) values, must be substantially different from the one based upon "true sugar" values. Since all investigations concerning the matter heretofore dealt only with apparent sugar values, we made it our task to obtain a more accurate image of the distribution of blood sugar by the determination of true sugar in the corpuscles and in the plasma.

Determination of True Sugar.

The true sugar was obtained by the indirect method as in our earlier work; *viz.*, as the difference between apparent sugar and reducing non-sugars. Having recognized that the precipitation of proteins in the presence of yeast in no way enhances the removal of the sugar—as stated also in a recent paper by Benedict (2)—we reverted to the older practice, employed by Rona and Takahashi (3), and recently by Folin and Svedberg (4), of fermenting the deproteinized filtrates. Thus we determine the apparent sugar and the reducing non-sugars in portions of one and the same filtrate. This technique yields values from 3 to 4 mg. per cent lower than our original procedure, a discrepancy which we are unable to explain definitely. At first thought we attributed the decrease to dilution by the water content of the yeast, but ex-

periments showed this not to be the case. If one and the same batch of yeast is used for consecutive determinations of the reducing non-sugars in two portions of a blood filtrate, the second determination, where dilution does not enter, yields no higher values than the first. Even more convincing than this were experiments with arabinose; the reduction exerted by solutions of this non-fermentable sugar was not lowered by treatment with yeast, showing that the dilution is entirely negligible. Apparently very little water is retained after centrifugation between the yeast cells, and the intracellular moisture obviously does not transude into the outer solution. From the fact that in a few pathologic specimens, after fermenting the whole blood, we found reduction values far in excess of the apparent sugar, we infer that some enzymes of the blood may produce non-fermentable reducing substances in contact even with washed yeast; this effect may prevail also in some normal specimens, only to such a small extent that it is hardly discernible in individual samples. Although the slight difference affects our results in no appreciable measure, we are inclined to accept as more accurate the lower values obtained from deproteinized filtrates.

Procedure.

The procedure is as follows: Into a Pyrex test-tube measure 6 to 7 cc. of a 20 per cent suspension of washed yeast; centrifuge and discard the supernatant water, invert the tube to drain for some seconds, and by means of a strip of filter paper remove the moisture adhering to the walls of the test-tube. Introduce 12 to 14 cc. of the deproteinized filtrate to be fermented, stir up the yeast with a glass rod, and allow to stand at room temperature for about 10 minutes with occasional inversions of the test-tube to prevent settling of the yeast. (Should the room temperature be unusually low, place the test-tube in a beaker containing water of 25–30°.) Transfer the contents to another Pyrex test-tube without moistening its walls above the final level, and centrifuge. Decant the supernatant fluid at once. This procedure without filtration yields over 10 cc. of perfectly clear sugar-free solution, sufficient for two determinations of its reduction value.

Such quantities of sugar as are ordinarily encountered in blood

filtrates are completely fermented at room temperatures within 5 minutes, but prolongation even for several hours of the contact between yeast and filtrate has no ill effect upon the results. On protracted incubation at body temperature, however, even the most carefully washed yeast is prone to yield non-fermentable reducing substances. Their amount is usually small, yet measurable at the end of 30 to 60 minutes.

The careful washing of the yeast must again be stressed as an essential phase of this technique. The impurities contained in commercial yeast represent a variable, complex source of error, and are probably responsible for the major part of the obscurity and confusion that has surrounded the problem of "residual reduction." A recent report by Bigwood and Wuillot (5) seems to bear out this contention. They determined the non-fermentable reducing substances in plasma after fermenting their specimens with unwashed commercial yeast for 30 minutes at body temperature, using blanks—carried through identical conditions—to correct their results. They report values between a trace and 26.1 mg. per cent in terms of glucose. These figures are undoubtedly wrong, and the main source of error is probably the action of plasma enzymes upon the soluble impurities of the yeast.

Regarding the application of *kaolin*, Rona and Takahashi (3) reported in 1911 that deproteinized blood filtrates, treated repeatedly with kaolin subsequent to fermentation, were optically neutral and contained no reducing substances; the non-fermentable reducing substances were obviously adsorbed by the kaolin in this procedure. Benedict (2) recently investigated the effect of kaolin, and found it to adsorb about 2 mg. per cent of the non-fermentable reducing substances. Our values are from 5 to 10 mg. per cent, the difference between Benedict's results and ours probably being due to the fact that we are using copper reagents of maximum reduction value. Paradoxically, the Folin-Svedberg technique, in which kaolin is employed, yields in many instances appreciably higher reducing non-sugar values than our procedure. A plausible explanation of this is that a fraction of the reducing impurities introduced with the yeast is left behind, which exceeds the fraction of reducing non-sugars adsorbed by the kaolin.

Sugar Content of Corpuscles.

In 1885 Otto (6), the first investigator concerned with the distribution of sugar in blood, asserted that the corpuscles are entirely devoid of sugar, the whole of the blood sugar being confined to the plasma. Corroborated by Abderhalden (7), this view was generally accepted until in 1909 Rona and Michaelis (8), and since then many other workers, demonstrated that the corpuscles of human blood always contain more or less fermentable sugar. But soon afterwards Bang and his coworkers (9, 10), and later, in 1919 Falta and Richter-Quittner (11), and quite recently Glassmann (12) offered evidence of the contrary effect, upholding Otto's conception. None of these authors attempted to explain this remarkable conflict of experimental results, but the conclusions of Falta and Richter-Quittner were a few years later summarily dismissed as erroneous in a report from Falta's own laboratory (13).

An inquiry into the cause of this controversy directed our attention to the fact that the authors asserting that the corpuscles are free of sugar, washed the corpuscles with several changes of saline in order to remove the adhering plasma preceding the determination of their sugar content. Examining the effect of such washings we ascertained that erythrocytes, mixed with 10 volumes of saline but for a minute, loose practically their entire sugar content to the fluid medium, and the reducing substances still present in them after centrifugation are—except for 3 to 5 mg. per cent—non-sugars. (Ege and Hansen (14) assumed on the ground of similar experiments that this latter reduction value represents a rather firmly "combined" fraction of the corpuscle sugar, while another fraction which is readily detached from the cells by rinsing, is loosely adsorbed upon the surface of the cells.) From this it is evident that the washing of the corpuscles is the error responsible for the false conclusion that the corpuscles are free of sugar.

The small amount of intercellular plasma, left with the corpuscles after centrifugation, has no measurable effect upon the result in the determination of corpuscle sugar. But there is a serious source of error, especially in the case of specimens containing no anticoagulants. In defibrinated blood the sum of

plasma sugar and corpuscle sugar is 5 to 10 mg. per cent below the sugar content of the whole blood as determined immediately before it is centrifuged. This discrepancy is due to glycolysis which—under the given circumstances—occurs in the main at the expense of the corpuscle sugar. Consequently, we accepted in the course of the present work the indirect determination of the corpuscle sugar as correct. The sugar is determined in the whole blood and in the plasma or the serum from which, with the knowledge of the corpuscle volume, the corpuscle sugar is calculated according to the practice followed by most investigators. As a control, the direct determination of the corpuscle sugar was also carried out.

Effect of Anticoagulants.

The experiments reported in this paper were all performed on defibrinated blood samples. The comparison of our results with those of Folin and Berglund (15), and of other authors who had taken every possible precaution to preclude alterations in the distribution of the blood sugar (collection of the blood in paraffined tubes without anticoagulants, *etc.*), proves to our satisfaction that defibrination does not alter the distribution. Nor have moderate amounts of anticoagulants any effect upon it, as we found identical distribution ratios in several portions of the same specimen, whether the coagulation was prevented by defibrination or by the addition of 0.1 to 0.15 per cent of potassium oxalate or sodium citrate. However larger quantities of these anticoagulants, which render the plasma appreciably hypertonic and cause the corpuscles to shrink, exert a marked influence upon the distribution of the sugar.

Sodium fluoride, which inhibits the action of yeast, must not be used in experiments involving the determination of true sugar values.

Determination of Distribution of Sugar.

Taking into account the foregoing considerations our ultimate course in the determination of the distribution of sugar is as follows: 20 to 25 cc. of blood are drawn from an arm vein of the subject. Promptly after defibrination two portions of 2 cc. each are measured out and deproteinized according to the Folin-Wu

method, while the remainder is being centrifuged at 3000 R.P.M. for 10 minutes. After syphoning off the serum, 5 cc. of the corpuscles are laked in 24 cc. (4.8 volumes) of water, and precipitated by the addition of 10 cc. (2 volumes) of 0.75 N sulfuric acid and 11 cc. (2.2 volumes) of 10 per cent sodium tungstate. For the determination of apparent sugar in plasma, two 1 cc. portions are deproteinized at 1:10 dilution, while for fermentation a 1:4 filtrate is prepared from 4 to 5 cc. of plasma, as the amount of the reducing non-sugars at 1:10 dilution is too low to be determined by the method employed throughout these experiments (16).

The apparent sugar values for corpuscles, as given in Tables I and II, are calculated from blood sugar and serum sugar. The reducing non-sugars of the corpuscles, too, represent calculated values, which are 3 to 5 mg. per cent higher than obtained by direct determination. The reason for this discrepancy is twofold; first, unlike the case of the apparent sugar, here the diluting effect of adhering plasma is appreciable (5 per cent of admixed plasma causes a decrease of almost 2 mg. per cent), and second, we find that the reducing non-sugar values slowly decline if blood or corpuscles are allowed to stand for some length of time, the loss mounting occasionally to 30 to 40 per cent after 4 to 5 hours incubation. Consequently, for corpuscles we accepted the calculated values of the reducing non-sugars as more accurate, although the difference between these and the directly determined values is too small to affect the results materially.

Table I presents the distribution of the sugar in blood specimens obtained from thirty-six healthy adults, thirty-two men and four women. As can be seen, the apparent sugar values conform with the generally accepted view of a nearly equal distribution of the blood sugar between the corpuscles and the plasma, the ratio, $\frac{\text{corpuscle sugar}}{\text{plasma sugar}}$ being on the average 110:100 (Frank (17), Bailey (18), Schmid (19), Folin and Berglund (15), John (20), Wiechmann (21), Ege and Hansen (14), and several others). But the actual ratio, based upon true sugar values, is quite different, to wit: the average is 77:100, with considerable deviations on either side; 70:100 may be considered the lowest ratio, a few high values approaching unity. High ratios are relatively infrequent as only in one-fourth of the cases were they above 80:100.

TABLE I.

Distribution of Sugar and of Reducing Non-Sugars in Human Blood.

Specimen No.	Apparent sugar.		Reducing non-sugars.		True sugar.		Corpuscle sugar / Serum sugar	
	Mg. in 100 cc. of:							
	Corpuscles.	Serum.	Corpuscles.	Serum.	Corpuscles.	Serum.	For apparent sugar.	For true sugar.
1	117	102	42	8	75	94	1.15	0.80
2	122	107	45	7	77	100	1.14	0.77
3	107	80	44	8	63	72	1.34	0.87
4	114	110	36	7	78	103	1.04	0.76
5	130	110	42	7	88	103	1.18	0.86
6	111	109	39	7	72	102	1.02	0.71
7	107	93	40	8	67	85	1.15	0.79
8	81	73	38	8	43	65	1.11	0.66
9	102	94	42	8	60	86	1.08	0.70
10	114	104	39	9	75	95	1.10	0.79
11	103	103	36	7	67	96	1.00	0.70
12	101	82	39	8	62	74	1.23	0.84
13	119	110	39	8	80	102	1.08	0.78
14	112	117	38	8	74	109	0.96	0.68
15	103	99	39	9	64	90	1.04	0.71
16	143	143	45	8	98	135	1.00	0.73
17	111	82	40	7	71	75	1.35	0.95
18	100	100	38	9	61	91	1.00	0.68
19	151	149	45	7	106	142	1.01	0.75
20	125	121	40	8	85	113	1.03	0.75
21	114	107	41	7	73	100	1.07	0.73
22	107	98	43	7	64	91	1.09	0.70
23	148	146	43	7	105	139	1.01	0.76
24	103	89	43	7	60	82	1.16	0.73
25	108	101	38	8	70	93	1.07	0.75
26	110	86	37	7	73	79	1.28	0.92
27	118	111	34	7	84	104	1.06	0.80
28	145	137	38	8	107	129	1.06	0.83
29	112	99	36	9	76	90	1.13	0.84
30	105	100	37	6	68	94	1.05	0.73
31	110	100	39	8	71	92	1.10	0.77
32	118	89	36	8	75	81	1.25	0.93
33	110	103	38	10	72	93	1.07	0.77
34	98	94	35	8	63	86	1.04	0.73
35	117	102	42	8	75	94	1.15	0.80
36	118	106	42	9	76	97	1.11	0.78
Lowest			34	6			0.96	0.66
Highest			45	10			1.35	0.95
Average			40	8			1.10	0.77

The distribution seems to be unaffected by the nutritional state of the subjects, as blood specimens with high sugar content, drawn after meals, exhibit variations within the same range as those obtained before breakfast. As to the constancy or variability of the distribution in one and the same subject, we do not possess sufficient data to justify a conclusion; in a few persons we found considerable variations.

The ratio, $\frac{\text{corpuscle sugar}}{\text{plasma sugar}}$, then, is an illusory figure if based upon apparent sugar values. According to Table I its average value is over 40 per cent higher than the average actual ratio. The discrepancy between these two values is, however, variable and is determined by two factors. A simple equation will be helpful in the analysis of the rôle of these factors:

$$R = \frac{c + 5\,n}{p + n}$$

where R is the distribution ratio calculated from apparent sugar values, c is the true sugar in corpuscles, p the true sugar in plasma, n represents the reducing non-sugars in plasma, and $5\,n$, with a good approximation, the reducing non-sugars in corpuscles.

If in this equation n gradually diminishes, to the same extent the value of R approaches $\frac{c}{p}$, that is the actual ratio of distribution; if n finally becomes zero, as with Benedict's latest reagent (2), or is separately determined by our method and ruled out by subtraction, then $R = \frac{c}{p}$, the actual ratio of distribution. The colorimetric methods of the Folin-Wu type yield lower values for n than the methods involving iodometric titrations, hence they furnish somewhat lower R values.

The other factor that is apt to distort the true ratio of distribution, and to cause variations in its value to a still greater extent, is the alteration of the blood sugar level. For a given analytical method n may be considered as nearly constant. Now, if the blood sugar level, and consequently both c and p rise, the discrepancy between $\frac{c + 5\,n}{p + n}$ and $\frac{c}{p}$ gradually diminishes, and finally —at very high blood sugar levels—the difference becomes almost

negligible. If, on the other hand, the values of c and p decline, the lower they become, the more prominent will be the disguising effect of n. Finally in extreme instances, where the blood sugar would approach zero value—*e.g.* as the result of glycolysis or of insulin hypoglycemia—the reducing substances still present in both corpuscles and plasma would lead to a ratio of $R = \frac{5n}{n}$, which would mean that the corpuscles contain 5 times as much sugar as the plasma.

A few examples in Table II illustrate the significance of this point, although they are far from representing extreme cases.

TABLE II.

Specimen No.	Description of specimen.	Apparent sugar. Mg. in 100 cc. of: Corpuscles.	Apparent sugar. Mg. in 100 cc. of: Serum.	True sugar. Mg. in 100 cc. of: Corpuscles.	True sugar. Mg. in 100 cc. of: Serum.	Corpuscle sugar / Serum sugar: For apparent sugar.	Corpuscle sugar / Serum sugar: For true sugar.
1	Diabetic blood	304	358	266	350	0.85	0.76
2	Normal blood	84	64	45	57	1.31	0.77
3	Blood from normal subject. Fasting	112	99	76	90	1.13	0.84
4	Blood from normal subject. 30 min. after glucose	184	184	148	175	1.00	0.85
5	Blood from normal subject. 3 hrs. after glucose	77	59	41	50	1.31	0.82

If the distribution of the sugar in Specimens 1 and 2 is compared on the basis of the apparent sugar values, the relative sugar content of the corpuscles in Specimen 2 appears to be $\frac{(1.31 - 0.85)\ 100}{0.85} =$ 54 per cent higher than that in Specimen 1, while the true sugar values plainly demonstrate that their ratios of distribution are practically identical. Without the knowledge of the true sugar values one is prone to arrive at the conclusion that the capacity to combine with sugar is impaired in the corpuscles of diabetic blood (Rona and Sperling (22), Loewi and collaborators (23), and others).

Specimens 3, 4, and 5 show the changes of the sugar in the

blood of a healthy man after the ingestion of 120 gm. of glucose. The apparent sugar values would indicate a decline of 13 per cent in the relative sugar content of the corpuscles as the sugar level goes up, and again an increase of 31 per cent as the sugar reaches a low level. Such figures are conducive to the assumption that in alimentary hyperglycemia, too, the distribution of the blood sugar is changed, inasmuch as the relative sugar content of the plasma becomes greater than at normal sugar levels (Höber (24), Wiechmann (21), and several others). The true sugar values, however, evince the fact that the actual ratio of distribution is practically the same in all of the specimens.

These findings suggest the desirability of a revision of some conclusions and theories derived from changes observed in the distribution of the blood sugar.

SUMMARY.

The distribution of the sugar between the corpuscles and the plasma in normal human blood was determined upon the basis of true sugar values. It is demonstrated that the ratio, $\frac{\text{corpuscle sugar}}{\text{plasma sugar}}$, as obtained from true sugar values, differs substantially from the ratio derived from apparent sugar values. This is an instance in which the substitution of apparent sugar for true sugar entails serious errors and misconceptions.

A modification is presented of the author's technique for the determination of reducing non-sugars by means of washed yeast.

BIBLIOGRAPHY.

1. Somogyi, M., *J. Biol. Chem.*, 1927, lxxv, 33.
2. Benedict, S. R., *J. Biol. Chem.*, 1928, lxxvi, 457.
3. Rona, P., and Takahashi, D., *Biochem. Z.*, 1910, xxx, 99.
4. Folin, O., and Svedberg, A., *J. Biol. Chem.*, 1926, lxx, 405.
5. Bigwood, E. J., and Wuillot, A., *Compt. rend. Soc. biol.*, 1927, xcvi, 414.
6. Otto, J. J., *Arch. ges. Physiol.*, 1885, xxxv, 467.
7. Abderhalden, E., *Z. physiol. Chem.*, 1898, xxv, 67.
8. Rona, P., and Michaelis, L., *Biochem. Z.*, 1909, xvi, 60.
9. Lyttkens, H., and Sandgren, J., *Biochem. Z.*, 1910, xxvi, 382.
10. Bang, I., *Biochem. Z.*, 1912, xxxviii, 166.
11. Falta, W., and Richter-Quittner, M., *Biochem. Z.*, 1919, c, 148.
12. Glassmann, B., *Z. physiol. Chem.*, 1926, clviii, 113.

13. Högler, F., and Ueberrack, K., *Biochem. Z.*, 1924, cxlviii, 150.
14. Ege, R., and Hansen, K. M., *Acta med. Scand.*, 1927, lxv, 279.
15. Folin, O., and Berglund, H., *J. Biol. Chem.*, 1922, li, 213.
16. Somogyi, M., *J. Biol. Chem.*, 1926, lxx, 599.
17. Frank, E., *Z. physiol. Chem.*, 1910, lxx, 129.
18. Bailey, C. V., *Arch. Int. Med.*, 1919, xxiii, 455.
19. Schmid, F., *Compt. rend. Soc. biol.*, 1922, lxxxvii, 1367.
20. John, H. J., *Arch. Int. Med.*, 1923, xxxi, 555.
21. Wiechmann, E., *Z. ges. exp. Med.*, 1924, xli, 462.
22. Rona, P., and Sperling, M., *Biochem. Z.*, 1926, clxxv, 253.
23. Loewi, O., *Klin. Woch.*, 1927, vi, 2169.
24. Höber, R., *Biochem. Z.*, 1912, xlv, 207.

HUMAN MILK STUDIES.

V. A QUANTITATIVE COMPARISON OF THE ANTIRICKETIC FACTOR IN HUMAN MILK AND COW'S MILK.*

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The prevalence of rickets in babies, as shown by the recent investigation conducted by the United States Children's Bureau (1), makes it of importance to know to what extent the milks generally recommended in infant feeding can justly be relied upon to supply the antiricketic factor. This is particularly pertinent when it is realized that rickets occurs at the period of development when milk constitutes the greater part, if not the whole, of the diet. It might be assumed that nature would provide an abundance of all essential growth-promoting factors in the pabulum for the young of all species; but clinical experience has demonstrated that under the present living conditions neither human milk nor cow's milk can always be relied upon to protect babies against rickets.

With the advent of the study of experimental rickets in animals, various attempts have been made to evaluate the antiricketic potency of human milk and cow's milk.

Steenbock and associates (2), using their ricketic ration, No. 2965, found that for cow's milk the minimum daily requirement for healing of rickets in rats was 12 cc. Honeywell, Dutcher, and Dahle (3) corroborated these results, using the same ration in a preventive type of experiment. Luce (4), however, demonstrated that 2 to 5 cc. of milk from cows kept on pasture were potent in preventing rickets when fed to rats on the McCollum ration, No. 3143, whereas 15 to 20 cc. of milk were ineffective if the cow was kept in the dark on either dry or green food.

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* A preliminary report of this investigation was presented before the Biological Chemistry Section of the American Chemical Society in Detroit, September, 1927.

In studying human milk, Lesné and Vagliano (5) concluded that this important nutrient contained no antiricketic properties. They fed, however, a maximum of 5 per cent of the ether extract of breast milk to rats with the Sherman-Pappenheimer ricketogenic ration. Kennedy and Palmer (6), on the other hand, later demonstrated that a level of at least 8 per cent of human milk fat was necessary for the production of a distinct line test in rats made ricketic by the McCollum ration, No. 3143. Hess (7) has reported that whereas 25 cc. of human milk daily ordinarily failed to cure ricketic rats on the Sherman-Pappenheimer diet, this same amount could be made effective by treating the mother with ultra-violet light.

In a recent publication, Hess (8) has presented data on the antiricketic factor of these two milks when fed in a preventive type of experiment. He states that "when from 20 to 25 cc. (cow's milk) were added to the standard ration, the development of rickets was prevented" and that "from 25 to 30 cc. of human milk had to be added to this ration to confer protection."

It is impossible to explain the conflicting results of the studies reviewed above, since environmental factors surrounding the lactating individual and variations in experimental technique of feeding the milk play important rôles in the analysis of the results. The experiments reported here were started in March, 1926, for the purpose of making a more detailed comparison of the antiricketic properties of human milk[1] and cow's milk which have been produced under definite conditions and fed to rats with an experimental technique somewhat modified from the heretofore approved methods.

EXPERIMENTAL.

Technique.

It has been demonstrated repeatedly that in order to produce rickets *in rats* at least two conditions must exist; namely, the absence of the so called antiricketic factor, and the inclusion in the ration of a suitable salt mixture containing 4 or 5 parts of calcium to 1 part of phosphorus. It has likewise been shown (11–13) that the addition of more phosphorus to such rations results in the prevention or the arrest of the disease. Yet from a survey of the literature it would seem that this latter requirement has not always been fully appreciated. Throughout the

[1] The information obtained here on the antiricketic potency of human milk, together with the experiments published heretofore (9, 10), form the foundation for biological studies on the effect of diet upon the vitamin content of human milk.

period of rickets development the ratio of Ca:P = 4 or 5 has been closely adhered to, but during the curative régime food substances rich in phosphorus or calcium have been superimposed on the ricketic diet without corresponding deductions being made in the salt composition of the basal ration. This correction is essential, particularly when large quantities of cow's milk are fed. The addition of milk to a ricketogenic diet may result in an intake of calcium and phosphorus in a ratio that would tend to cause a healing of the bone lesion, even in the absence of the antiricketic factor. This criticism applies to any of the ricketogenic rations in general use. The primary consideration throughout this entire study, then, has been to maintain a ratio of Ca: P = 5 during the ricketogenic and curative periods, and to eliminate possible bone-calcifying agents other than the antiricketic.

Vigorous young rats, 21 to 23 days old, reared by mothers on the standard breeding ration,[2] were employed. They were placed in individual cages and on raised screens to prevent ingestion of feces and a consequent disturbance in the calcium: phosphorus ratio in the dietary. No attempt was made to keep the animals in absolute darkness; they were, however, placed in cages in a dark corner farthest from the windows, and at no time were exposed to the direct rays of the sun. The ricketic rations were fed *ad libitum*, accurate records of the weekly food intake being kept. Additions to the basal ration, *i.e.* milk or butter fat, were made daily in separate dishes. Fresh distilled water was available in the drinking bottles at all times except when the rats refused to drink the entire amount of milk allotted to them. The animals were held on the ricketogenic rations for a minimum period of time, to avoid the production of advanced pathological lesions. The occasional rat failing to grow on account of inadequate appetite has been discarded from the experiment.

For these experiments the Osborne, Mendel, and Park ration (14) was adopted, and somewhat modified to meet the requirements of availability and low cost of food materials. The ad-

[2] The breeding ration consisted of the Sherman diet of two-thirds whole wheat, one-third whole milk powder, and 1.3 per cent NaCl, together with daily supplements of fresh cabbage or lettuce, and fresh cow's milk *ad libitum*.

vantage of this synthetic ration is that the mineral constituents[3] are present in approximately the same proportion as in the Osborne and Mendel standard Salt Mixture IV with the exception of calcium and phosphorus, and that these two can be added separately in any amount and in any ratio desired. From the total calcium and phosphorus required, deduction has been made for the calcium and phosphorus content of the other constituents of the ration, and the balance added in the form of $CaCO_3$ and H_3PO_4, previously mixed and dried. Butter fat was given daily so that the rats would not experience a shortage of vitamin A. It was assumed that approximately 1 per cent of butter fat would not prevent the production of rickets, since McCollum (15) found that only slight healing took place when 15 to 30 per cent was fed for 14 days. The modified ricketic ration has the following composition:

	per cent		
$CaCO_3$	1.55	Calcium	0.622 gm.
H_3PO_4 (85 per cent)	0.10	Phosphorus	0.125 "
Salt Mixture XXX[3]	2.60	Ratio Ca:P =	5
Yeast	6.00		
Egg albumin[4]	18.00		
Dextrin	71.75		
Butter fat	3 drops daily.		

This standard ricketogenic ration has been used in studies only when the food substances under investigation contained but inappreciable amounts of calcium and phosphorus.

[3] The authors desire to express their appreciation to Professors Osborne and Mendel for the privilege of using their ricketic salt mixture prior to its publication. The calcium- and phosphorus-free Salt Mixture XXX has the following composition:

	gm.		*gm.*
$MgCO_3$	21.80	Fe citrate·1½ H_2O	6.34
Na_2CO_3	30.10	KI	0.02
K_2CO_3	118.60	$MnSO_4$	0.079
HCl	95.30	NaF	0.248
H_2SO_4	9.20	$K_2Al_2(SO_4)_3$	0.0245
Citric acid·H_2O	30.60		

In addition 622 mg. of calcium and 125 mg. of phosphorus were added to every 100 gm. of ration. These amounts have been found by Osborne and Mendel to be adequate for the production of rickets in rats.

[4] Dried egg albumin was secured from T. M. Duché and Sons, 376–378 Greenwich Street, New York.

TABLE I.

Composition of Rations I and II Fed in Connection with Different Amounts of Human Milk and Cow's Milk.

Milk. Type.	Daily amount.	Period.	Ration No.	Constituents of ration. $CaCO_3$	H_3PO_4, 85 per cent.	Salt mixture.	Egg albumin.	Yeast.	Dextrin.	Butter fat daily.	Total Ca.	Total P.	Ca:P ratio.
	cc.			*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*drops*	*per cent*	*per cent*	
Human.	25*	Premilk-feeding.	I	1.90	0.19	2.6	18	6	71.1	3	0.770	0.154	5
Ca per cent = 0.030.	30	Milk-feeding.	II	1.63	0.00	2.6	18	3	74.8	0	0.770†	0.154†	4-5
P " " = 0.014.	35												
	40	Premilk-feeding.	I	2.30	0.30	2.6	18	6	70.8	3	0.925	0.185	5
		Milk-feeding.	II	1.30	0.00	2.6	18	3	75.1	0	0.925†	0.185†	4-5
	15	Premilk-feeding.	I	3.7	0.72	2.6	18	6	69.0	3	1.49	0.298	5
Cow's.		Milk-feeding.	II	2.6	0.00	2.6	18	3	73.8	0	1.49†	0.298†	4-5
Ca per cent = 0.110.													
P " " = 0.097.	20	Premilk-feeding.	I	5.55	1.27	2.6	18	6	66.6	3	2.23	0.445	5
		Milk-feeding.	II	4.00	0.00	2.6	18	3	72.4	0	2.23†	0.445†	4-5
	30	Premilk-feeding.	I	6.50	1.50	2.6	18	6	65.4	3	2.55	0.51	5
		Milk-feeding.	II	4.60	0.00	2.6	18	3	71.8	0	2.55†	0.51†	4-5

* The difference in the calcium and phosphorus content of the 25, 30, and 35 cc. levels of human milk was so slight that Rations I and II were calculated for the 30 cc. series and used in the 25 and 35 cc. series as well.

† Approximately.

Variations of this standard ration have been used in succeeding studies in which different types and amounts of milk were fed. Each experiment was divided into a period of rickets development and a 7 day curative period of milk feeding. To avoid a marked change in the calcium and phosphorus intake of the rats during these two periods, it was necessary that for each proposed level of milk a separate ricketogenic ration should be prepared with an appropriate percentage of calcium and phosphorus. In addition to this, the basal food fed with milk during the curative period was so constructed that the sum total of the calcium and of the phosphorus eaten would still conform to the required ricketic ratio of 5:1. To this end, then, for every level of milk, two basal diets were prepared,[5] Ration I, the ricketogenic ration, and Ration II, fed only in connection with milk. These diets differed primarily in the percentage of calcium and phosphorus actually incorporated in them (Table I). In the rations fed in connection with human milk, calcium and phosphorus were present in approximately optimal amounts; whereas the high phosphorus content of the cow's milk necessitated the addition of abnormally large amounts of calcium to the ration.

During the milk feeding period, although theoretically calculated to be constant and the same for all animals, the calcium and phosphorus ratio has varied somewhat on account of a difference in the basal food intake of individual animals. In the analysis of the data secured in both the human milk and the cow's milk series, therefore, all records showing an actual calcium and phosphorus intake not falling within the limits of the ricketic ratios of 5:1 or 4:1 have been discarded.

In the examination for ricketic changes the animals were

[5] The following method has been used in the calculation of these rations. Ration I (*a*) the calcium and phosphorus content of the proposed total milk intake (7 days) was determined; (*b*) from previous studies an estimation was made of the amount of basal food that would be eaten in connection with the different quantities of milk; (*c*) an equivalent percentage of phosphorus was incorporated in the ration; (*d*) calcium was added to the extent of 5 times the amount of phosphorus. Ration II (*a*) no phosphorus was added, this being supplied largely in the milk; (*b*) the total calcium required in Ration I minus the calcium supplied in the milk was added in the form of $CaCO_3$. In both rations corrections were made for the content of calcium and phosphorus in the egg albumin and yeast.

etherized and roentgenographed. Autopsies were performed immediately after, and notations were made on the condition of the costochondral junctions, the epiphyses of the long bones, and the thoracic and abdominal cavities. The bones of the hind legs were removed and both tibiæ were cut lengthwise, one-half of each being used at once for the silver nitrate "line test" of McCol-

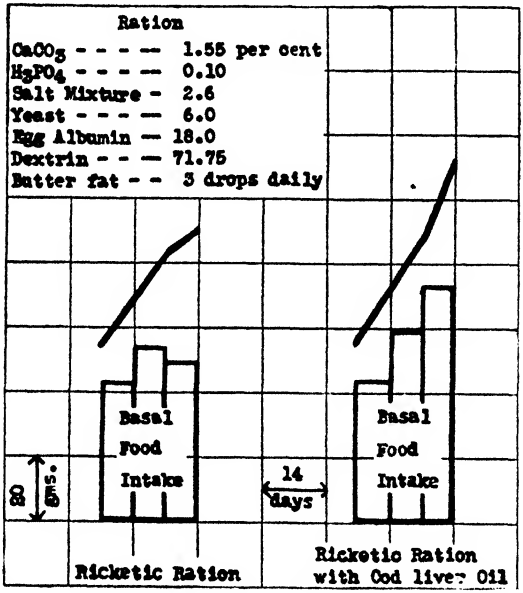

CHART I. The standard ricketogenic ration. Composite growth curves and food intakes of rats on the standard ricketogenic ration with and without cod liver oil.

lum *et al.* (16) and the other portion preserved in formalin for histological examination. The femora were set aside for chemical analyses,[6] determinations being made for each rat in order to study the individual variations of animals on the same ration.

[6] Chemical analyses were made on the dry, fat-free femora, the muscle tissue having been previously removed by peeling the periosteum from the shaft. The bones, slit at each end, were dried to constant weight at 100°, and extracted first with hot 98 per cent alcohol for 24 hours, and then with anhydrous ether for the same length of time. They were then dried, weighed, and ashed in a muffle furnace at a temperature of 500–600°. The

TABLE II.

Type and Degree of Bone Condition of Rats Fed 21 Days on Standard Ricketic Ration with and without Cod Liver Oil.

Rat No.	Increment in weight per wk.	Food intake per wk.	Cod liver oil.	Final age.	Line test.			Histological examination.			X-ray diagnosis.	Bone analysis.			Ash in fat-free dried feces.
					Cartilage band.	Osteoid tissue.	Calcification (provisional zone).	Cartilage cells.	Osteoid tissue.	Calcification (provisional zone).		Total ash.	Ca	P	
	gm.	*gm.*	*days*	*days*								*per cent*	*per cent*	*per cent*	*per cent*
1028	11	52	None.	42	Very wide and irregular.	Moderate amount.	Negative.	Many prongs extending into metaphysis; irregular arrangement.	Irregular formation, uncalcified.	None.	Severe rickets.	28.3	10.6	5.0	27.7
1029	9	44	"	42	Moderate widening.	" "	"	" "	" "	"	" "	31.3	11.8	5.3	25.7
1030	11	49	"	42	" "	Small amount.	"	" "	" "	"	" "	31.3	11.6	5.3	28.7
1032	13	56	"	42	" "	" "	"	" "	Uncalcified narrow metaphysis.	"	" "	32.2	12.2	5.5	28.2
1033	9	43	"	42	Very wide and very irregular.	Large amount.	"	" "	Irregular formation, wide metaphysis, uncalcified.	"	" "	28.2	10.3	4.7	27.3

1034	10	52	None.	42	Moderate widening.	Small amount.	Negative.	Many prongs extending into metaphysis; irregular arrangement.	Irregular formation, uncalcified.	None.	Severe rickets.	29.8	11.2	5.1	29.6
Average...	10	49										29.6	11.3	5.1	27.9
1035	9	54	21	42	Narrow, regular.	None.	Positive.	Normal arrangement.	None.	Heavy.	Approaching normal.	41.2	15.7	7.2	14.7
1036	15	54	21	42	" "	"	"	" "	"	"	" "	37.5	14.1	6.8	14.5
1037	17	68	21	42	" "	"	"	" "	"	"	" "	39.2	14.7	6.9	16.0
1038	17	64	21	42	" "	"	"	" "	"	"	" "	40.6	15.3	7.4	11.7
1039	13	54	21	42	" "	"	"	" "	"	"	" "	43.6	16.9	7.8	11.6
1040	8	44	21	42	" "	"	"	" "	"	"	" "	39.3	14.3	6.8	15.5
Average...	13	56										40.2	15.2	7.1	14.0

* This ration contains 0.622 per cent Ca and 0.125 per cent P.

Growth and Deportment of Rats on Ricketic Rations.

Each ricketogenic ration, No. I, irrespective of its calcium and phosphorus content, has allowed for continued growth and avid appetite in young rats. By the end of the 3rd week, however, the result of the deprivation of the antiricketic factor has manifested itself in a retarded rate of growth (see Chart I). Enlargement by the epiphyseal ends of the long bones has been markedly noticeable by the 10th day. Uncomplicated rickets has appeared as early as the 14th to the 21st day as judged by various methods in current use. Pathological changes, such as marked curving of the legs and spine, inability to stand, *etc.*, have not been produced in this short experimental period. Xerophthalmia and respiratory diseases have not been observed.

In Table II data are presented on the behavior of a typical group of rats receiving the standard ricketic ration with and without the addition of cod liver oil. The diagnosis of rickets has been confirmed by three different methods; *i.e.*, histological, line test, and x-ray examinations. Additional evidence has been found in the percentages of calcium, phosphorus, and ash of the bones. These chemical analyses have been found to be of great value in determining the *degree* of calcification of bones, but should not be relied upon alone in the diagnosis of rickets in rats. As shown by Chick, Korenchevsky, and Roscoe (19), calcium and phosphorus determinations based on the ash content of bones are of no significance, these values remaining approximately constant, irrespective of diet, sex, or age of the animal. Although complete metabolism studies were not possible, interesting data have been obtained on the ash content of feces[7] of rats on these dietaries. On the standard ricketic ration a fecal ash of approximately 28 per cent was excreted, whereas on the ricketic rations, containing four times as much calcium and phosphorus, 60 per cent ash was excreted.

weighed ash was dissolved in hydrochloric acid, transferred to volumetric flasks, neutralized with NH_4OH, and made up to volume. Aliquot portions were used for the determination of calcium by the Clark method (17) and for phosphorus according to the procedure of Fiske and Subbarow (18).

[7] In the determination of fecal ash, the feces were extracted and ashed in the same manner as described for bone analysis.

Human Milk and Cow's Milk as Sources of the Antiricketic Factor.

Raw human milk was obtained fresh daily from the Detroit Bureau of Wet Nursing and was representative of the pooled milk from ten to sixteen women on the average American dietary. A survey indicated that these wet nurses had not taken cod liver oil during the periods of lactation under investigation. Various samples of the milk contained, as an average, 30 mg. of calcium and 14 mg. of phosphorus per 100 cc. The fat content of the milk ranged between 2.7 and 3.5 per cent. The certified cow's milk[8] was obtained from a herd of 450 animals fed a concentrate of grains, bone meal, and bran in addition to ensilage and alfalfa hay. The cows were kept in light, well ventilated stalls, but had no direct exposure to sunshine and little access to green pasture. The calcium and phosphorus contents were found to be respectively 100 and 97 mg. per 100 cc. The fat contents averaged 4 per cent.

As shown by the line test of the tibiæ of the litter mate controls, the rats in the human milk series were ready for curative feeding after 21 days on the ricketogenic rations, No. I. At this time they were changed to rations, No. II, and given human milk in quantities of 25, 30, 35, and 40 cc. daily. Since rats do not eat human milk avidly, it was necessary in the latter series to condense the milk under partial vacuum to about ⅓ of the original volume. When this was done, the rats were able to consume all the milk given them. After 7 days, the animals were killed with ether and examined in detail. Pronounced beading of the ribs, enlargement of the joints, and softness of the bones were found. On treating the freshly cut tibiæ with silver nitrate, it was demonstrated that no calcium phosphate had been deposited in the provisional zone of calcification at the base of the wide and irregular bands of cartilage. This was in direct contrast to the picture presented by the animals receiving for the same length of time 5 drops of cod liver oil daily in addition to milk and Ration II. Here the cartilage band had become narrower and straighter and showed a heavy precipitation of calcium salts at its base; *i.e.*, a positive line test. Analyses of the femora of rats receiving different amounts of milk showed no increase in the percentage of

[8] The cow's milk was obtained from the Walker-Gordon Laboratories of the Detroit Creamery Company.

TABLE III.

*Comparative Data on the Antiricketic Properties of Human and Cow's Milk.**

Kind of milk.	Ricketogenic period.			Curative period (7 days).				Final age.	Line test.		X-ray.	Analysis of bones.		
	Time on ration.	Increment in weight per wk.	Food intake per wk.	Milk daily.	Cod liver oil daily.	Basal food intake.	Increment in weight.		Cartilage band.	Calcification of provisional zone.		Ash.	Ca	P
	days	*gm.*	*gm.*	*cc.*	*drops*	*gm.*	*gm.*	*days*				*per cent*	*per cent*	*per cent*
Human milk.	35	8	45					56	Wide, irregular.	Negative.	Rickets severe, active.	28.1	10.1	4.5
	28	10	48	25	0	30	9	56	" "	"	" "	27.7	9.7	4.1
	28	9	43	25	5	29	2	56	" slightly irregular.	Positive.	Rickets undergoing healing.	28.2	9.9	4.4
	21–28	10	49					42–49	Wide, irregular.	Negative.	Rickets, moderate to severe.	30.5	10.8	4.8
	21	11	51	30	0	34	9	49	" "	"	" "	29.7	10.2	4.6
	21	10	49	30	5	31	7	49	" regular.	Positive.	Rickets undergoing healing.	33.5	11.3	5.1
	21–28	9	44					42–49	Wide, irregular.	Negative.	Rickets, moderate.	32.8	11.6	5.2
	21	11	50	40	0	30	4	49	" "	"	" "	32.9	11.4	5.2
	21	9	55	40	5	24	3	49	" regular.	Positive.	Rickets undergoing healing.	35.3	10.9	5.1

Cow's milk.	32	2	27					53	Wide, irregular.	Negative.	Rickets.	30.4	10.2	4.8
	32	12	39	10†	0			53	" "	"	"	30.6	10.6	4.9
	32	10	35	10†	5			53	Narrow, regular.	Positive.	Normal bone.	47.5	17.3	8.5
	16–28	8	43					37–49	Wide, irregular.	Negative.		30.7	11.1	5.0
	16–21	10	43	30	0	26	14	42–49	" regular.	Positive.		40.8	15.1	7.1
	16–21	8	41	30	5	26	8	42–49	" "	"		39.8	15.3	7.3

* Results presented are the averages for each group.

† Milk-fed throughout the study as a prophylaxis.

calcium, phosphorus, or total ash due to the addition of milk. The data in Table III show conclusively that there was no antiricketic factor present in 25, 30, or 40 cc. of the human milk fed in these experiments.

On the high calcium and phosphorus ricketogenic rations of the cow's milk series, it was found that rats could not be maintained in an unhealed ricketic condition beyond a period of 28 to 35 days. Since typical rickets was produced as early as the 14 th day, curative feeding was instituted at about this time. When 30 cc. of cow's milk were given daily for a period of 7 days, very definite healing of the bone lesion was found in all cases (Table III). The degree of calcification appeared to be as great as that induced by cod liver oil for the same length of time. The antiricketic properties of 5 cc. or of 10 cc., however, were so negligible that no beneficial effect was observed when these small amounts were fed daily to rats in a series of preventive experiments.[9]

In analyzing the results of these experiments it was observed that in some cases there was a lack of correlation of the x-ray and line test pictures with the ash content of the bones. The feeding of cod liver oil for 7 days to rats made ricketic on rations low in calcium and phosphorus, has resulted in the deposition of calcium phosphate at the epiphyseal line, as shown by the line test and x-ray pictures, without appreciably influencing the ash content of the bone. On the other hand, when calcium and phosphorus were present in large amounts in the food, the addition of cod liver oil for 7 days resulted not only in a positive line test but also in an increased percentage of ash in the femora. It is thus apparent that a level of food calcium and phosphorus of 0.622 and 0.125 per cent respectively, is too low to allow for the deposition of calcium

[9] These experiments were conducted early in 1926 in cooperation with Dr. I. McQuarrie of the Henry Ford Hospital to determine whether or not the antiricketic potency of milk could be increased by irradiating cows with ultra-violet light. For this purpose four cows were kept in stalls under identical experimental conditions with the exception that while two of them had access to light only as it came through ordinary window glass, the other two were exposed to the rays of a quartz mercury vapor lamp at a distance of 36 inches for 1 hour daily. This was done for 3 months prior to starting feeding experiments with rats. In the small amounts of these milks fed (5 to 10 cc.) daily for 28 days to young rats on an appropriately constructed ricketic ration, no protection was afforded against rickets.

salts in the shaft of the bone when cod liver oil is fed for a short period of 7 days. Whereas when 2.55 per cent of calcium and 0.51 per cent of phosphorus are present in the ration, cod liver oil feeding is attended by the deposition of calcium salts not only at the epiphyseal-diaphyseal junction but also in the shaft of the bone.

These experiments demonstrate that for rats human milk contains no demonstrable antiricketic factor, whereas cow's milk does possess a rickets-healing substance. Since the Ca:P ratio of the dietary has been carefully controlled during the period of milk feeding, and since no appreciable change in the calcium and phosphorus level has occurred during the entire experimental period, it can be stated that the curative properties of cow's milk are not due to the large percentage of calcium and phosphorus present in the milk. It may be concluded, furthermore, that this factor in cow's milk is analogous to the bone-calcifying properties of cod liver oil.

SUMMARY.

1. On a modified Osborne and Mendel synthetic ricketogenic ration, growth and appetite of rats have been satisfactory. Rickets has developed as early as 14 to 21 days.

2. By varying the calcium and phosphorus content of the ration, it has been possible to maintain an approximately constant ratio of Ca:P = 5 throughout the rickets-developing period and the curative period of milk feeding.

3. Data are presented which show that human milk fed in amounts of 25, 30, or 40 cc. daily contains no antiricketic factor.

4. Under the same carefully controlled conditions, 30 cc. of cow's milk fed daily for 7 days induced marked healing of ricketic lesions in rats.

The authors wish to express their appreciation of the interest and cooperation of Dr. Lawrence Reynolds, director of the X-ray Department, at the Children's Hospital of Michigan, who has made the roentgenological diagnoses for this study.

BIBLIOGRAPHY.

1. Eliot, M. M., *J. Am. Med. Assn.*, 1925, lxxxv, 656.
2. Steenbock, H., Hart, E. B., Hoppert, C. A., and Black, A., *J. Biol. Chem.*, 1925, lxvi, 441.

3. Honeywell, H. E., Dutcher, R. A., and Dahle, C. D., *J. Biol. Chem.*, 1927, lxxiv, p. lxxvii.
4. Luce, E., *Biochem. J.*, 1924, xviii, 716.
5. Lesné, E., and Vagliano, *Compt. rend. Soc. biol.*, 1924, xci, 143.
6. Kennedy, C., and Palmer, L. S., *Proc. Soc. Exp. Biol. and Med.*, 1925, xxiii, 230.
7. Hess, A. F., Weinstock, M., and Sherman, E., *J. Am. Med. Assn.*, 1927, lxxxviii, 24.
8. Hess, A. F., and Weinstock, M., *Am. J. Dis. Child.*, 1927, xxxiv, 845.
9. Macy, I. G., Outhouse, J., Graham, A., and Long, M. L., *J. Biol. Chem.*, 1927, lxxiii, 175.
10. Macy, I. G., Outhouse, J., Graham, A., and Long, M. L., *J. Biol. Chem.*, 1927, lxxiii, 189.
11. Sherman, H. C., and Pappenheimer, A. M., *Proc. Soc. Exp. Biol. and Med.*, 1921, xviii, 193.
12. Shipley, P. G., Park, E. A., McCollum, E. V., and Simmonds, N., *Proc. Soc. Exp. Biol. and Med.*, 1921, xviii, 277.
13. Karelitz, S., and Shohl, A. T., *J. Biol. Chem.*, 1927, lxxiii, 665.
14. Osborne, T. B., Mendel, L. B., and Park, E. A., *Proc. Soc. Exp. Biol. and Med.*, 1923-24, xxi, 87.
15. McCollum, E. V., and Simmonds, N., The newer knowledge of nutrition, New York, 3rd edition, 1925, 404.
16. McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., *J. Biol. Chem.*, 1922, li, 41.
17. Clark, G. W., *J. Biol. Chem.*, 1921, xlix, 487.
18. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, lxvi, 375.
19. Chick, H., Korenchevsky, V., and Roscoe, M. H., *Biochem. J.*, 1926, xx, 622.

STUDIES ON RACEMIZATION.

VII. THE ACTION OF ALKALI ON CASEIN.

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In previous publications[1] from this laboratory it has been pointed out that the progress of racemization of a given protein at different hydrogen ion concentrations may lead to definite conclusions regarding certain details of the structure of the protein molecule. This expectation is based upon observations on the action of alkali upon peptides and upon ketopiperazines. Dipeptides are not appreciably racemized under the influence of alkali. Higher polypeptides undergo racemization only at a very slow rate. Ketopiperazines, on the other hand, are racemized very rapidly under the proper conditions, but when the concentration of alkali is such that the rate of hydrolysis of the ketopiperazines is very high, the extent of racemization is very low. Hence, if a protein molecule contained ketopiperazines, it would be expected to behave in the following way. On treatment with dilute alkali, it would undergo marked racemization; on treatment with stronger alkali the degree of racemization would be minimal. A result of this type was actually observed in the case of gelatin. Table I gives a summary of the data which were reported in a previous article.[2]

The results of the observations on the action of alkali on casein are not so easily interpreted as those in the case of gelatin. The

[1] Levene, P. A., and Pfaltz, M. H., *J. Biol. Chem.*, 1925, lxiii, 661; *J. Gen. Physiol.*, 1925, viii, 183; *J. Biol. Chem.*, 1926, lxviii, 277; 1926, lxx, 219. Levene, P. A., Bass, L. W., Steiger, R. E., and Bencowitz, I., *J. Biol. Chem.*, 1927, lxxii, 815. Levene, P. A., and Bass, L. W., *J. Biol. Chem.*, 1927, lxxiv, 715. Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, 1928, lxxvi, 299.

[2] Levene, P. A., and Bass, L. W., *J. Biol. Chem.*, 1927, lxxiv, 715.

progress of racemization under the influence of alkali in higher dilution (0.5 N and 1.0 N) is such as would be expected from a structure containing ketopiperazines. On treatment with more concentrated alkali (5.0 N), casein, in contrast to gelatin and to ketopiperazines, shows a still higher rate of racemization than with dilute alkali.

From Table IV and Fig. 1 it is seen that with 1.0 N sodium hydroxide casein in 24 hours undergoes 41 per cent racemization calculated on the basis of total possible racemization or 47 per cent on the basis of maximum observed racemization. This observation means that of all the amino acids which are linked in a manner permitting racemization, 47 per cent are racemized in 24 hours. Ketopiperazines which have thus far been studied undergo as much as 80 per cent racemization in the course of 24 hours. With 5.0 N sodium hydroxide casein in 24 hours undergoes 66 per cent racemization calculated on the basis of maximum observed racemization. This degree of racemization is of the order of magnitude of the racemization of ketopiperazines, but for the ketopiperazines thus far studied, the rate of hydrolysis under the influence of strong alkali is so high that racemization does not set in at all.

TABLE I.

Racemization of Gelatin by Sodium Hydroxide.

NaOH	Time.	Rotation.	Racemization.
	days	*degrees*	
0.1 N	Control.	−0.38	−
	1	−0.69	+
	2	−0.79	+
1.0 N	Control.*	−0.36	−
	1	−1.13	+
	2*	−0.96	+
3.0 N	Control.	−0.23	−
	1	−0.20	−
	2	−0.18	−
	4	−0.13	−

* Through a typographical error these experiments were indicated in the table in the original paper as 0.1 N NaOH. Levene, P. A., and Bass, L. W., *J. Biol. Chem.*, 1927, lxxiv, 724.

Hence it seems that casein is not composed of ketopiperazines of the type thus far studied, nor is it a simple polypeptide of the type thus far studied. Two alternative assumptions may be offered to explain the conduct of casein: first, that the ketopiperazines in casein are more stable than the ketopiperazines thus far studied; or, second, that the order of linkage of the amino acids is such as to facilitate racemization.

It is certain that in casein, as in ketopiperazines or in peptides, racemization precedes cleavage. This conclusion is warranted by the fact that the unhydrolyzed part of casein shows the same

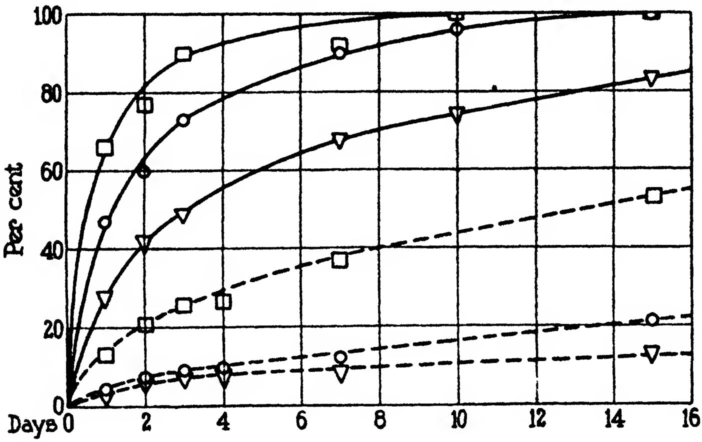

FIG. 1. The racemization and hydrolysis of casein by sodium hydroxide at 25°. The racemization curves are drawn in solid lines, the hydrolysis curves in broken lines. ▽ = 0.5 N NaOH; ○ = 1.0 N NaOH; □ = 5.0 N NaOH.

degree of racemization as the partially hydrolyzed part (caseoses), as seen from Tables IX and X.

The greater stability of casein as compared with gelatin is clearly seen from a comparison of Tables V and VI. Furthermore, as is seen from Table VII, it was not possible to increase the velocity of alkaline hydrolysis without increasing the rate of racemization. It was hoped that by raising the temperature of the reaction to 125° the rate of hydrolysis might be increased to such an extent that racemization would be avoided. However, under these conditions complete racemization was observed after 2 hours, whereas hydrolysis apparently was not yet complete in the same

interval of time. In this connection it may be recalled that Levene and Simms,[3] in their work on the relation of structure to the rate of hydrolysis of peptides, have found that peptides methylated on the nitrogen atom possess much greater stability than ordinary peptides.

Further work on synthetic peptides and on synthetic ketopiperazines, as well as work on a series of proteins of distinctly different composition, is required in order that the observations on the action of alkali on casein and on other proteins may be fully interpreted.

EXPERIMENTAL.

General Procedure.—The experimental procedure employed in the present investigation is essentially the same as that described in the previous paper. However, the hydrolyses were effected with hydrochloric acid instead of sulfuric acid because of the greater efficiency of the former. Likewise, these hydrolyses were carried out at a higher temperature in order to reduce the time necessary to reach a constant, maximum amino nitrogen ratio.

The protein employed was a uniform sample of Kahlbaum casein (Hammarsten). It gave the following analysis (calculated as dry material).

C 53.56, H 6.91, P 0.63, S 1.13, N 14.94, NH_3 0.80.
Ash 2.16. Moisture 8.41.

A uniform sample of norit was used as decolorizing agent.

The acid hydrolyses were carried out at 125° in a large glycerol thermostat equipped with a steam preheater and heated by an intermittent electric heating unit controlled by a thermoregulator. Thorough stirring was maintained by means of a motor. The temperature was constant within ± 0.5°.

The hydrolyses were carried out with 1.000 gm. (calculated as dry substance) of casein, 0.400 gm. of norit, and 10.0 cc. of standard acid, the mixtures being sealed in Pyrex test-tubes. After hydrolysis at 125° the solutions were filtered and diluted to 25.0 cc. The rotations of these solutions were measured in 4.00 dm. open

[3] Levene, P. A., Simms, H. S., and Pfaltz, M. H., *J. Biol. Chem.*, 1924, lxi, 445; 1926, lxx, 253. Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1924–25, lxii, 711.

tubes at 25° for the wave-length 5892 Å. All rotations were corrected to a concentration of 0.956 mg. of nitrogen per cc. in the solutions used for determining the amino nitrogen ratios, which were prepared by diluting 4.00 cc. of the rotation solutions to 25.0 cc.

Acid Hydrolysis at 125°.—A series of experiments with different concentrations of acid and different periods of heating was run in order to determine the optimum conditions for complete hydrolysis of the protein. In our choice of hydrochloric acid and a temperature of 125°, we were guided by the data on gelatin which we have reported previously.

Samples of casein equivalent to 1.000 gm. of dry substance with 0.400 gm. of norit and 10.0 cc. of standard acid were sealed in Pyrex test-tubes. The tubes were shaken to insure thorough mixing and were then heated for the required periods of time in the 125° thermostat.

The contents of the tubes were filtered through small folded filters into 25.0 cc. flasks and the filters were washed carefully with distilled water. In the experiments with 5.0 N acid, the flasks were then filled to the mark with distilled water, the resulting solutions thus being 2.0 N with respect to the acid. In the experiments with 1.0 N and with 3.0 N acid the necessary quantity of strong acid was added to bring the final 25.0 cc. solutions to a concentration of 2.0 N acid, in order that the rotations might be strictly comparable. The rotations of these solutions were then read.

For analysis, 4.00 cc. of the rotation solutions were neutralized to phenolphthalein with alkali and diluted to 25.0 cc. Total nitrogen was determined on 10.0 cc. samples (Kjeldahl) and amino nitrogen on 2.00 cc. samples (micro Van Slyke, 15 minutes).

The data are given in Table II. Each value is the mean of at least two independent experiments. The agreement between independent experiments was very good, the error in the rotations being ± 0.03° and in the amino nitrogen ratios ± 0.7 per cent in the solutions in which hydrolysis was complete or nearly complete. In the experiments with a lower degree of hydrolysis, the experimental error was greater. The degrees of hydrolysis recorded in Column 4 are calculated on the assumption that complete hydrolysis is represented by an amino nitrogen ratio of 76.2 per cent and

that the amino nitrogen ratio of the original material is 5.4 per cent.

A series of experiments with quantities of norit varying from 0 to 0.800 gm. showed that the use of this substance as a decolorizing agent introduced no appreciable error in the amino nitrogen ratios or in the rotations. (The rotations in the experiments with less than 0.200 gm. of norit could not be read because of their strong red color.)

An inspection of the data shows that hydrolysis is complete on heating with 5.0 N acid for 4 hours. These conditions were therefore adopted as standard conditions for the racemization experiments.

TABLE II.

Hydrolysis of Casein by Hydrochloric Acid at 125°.

Acid. (1)	Time. (2)	$\frac{\text{Amino N}}{\text{Total N}}$. (3)	Hydrolysis. (4)	α_D^{25} corrected. (5)
	hrs.	*per cent*	*per cent*	*degrees*
1.0 N	2	46.5	58.1	−4.15
	6	56.2	71.8	−1.15
	24	62.0	80.0	+0.50
3.0 N	2	65.5	84.9	+0.88
	6	72.0	94.1	+1.61
	24	76.4	100.3	+1.78
5.0 N	2	70.3	91.7	+1.60
	4	75.8	99.5	+1.86
	6	76.3	100.1	+1.80
	24	76.1	99.9	+1.75

The data for the calculation of the rotation of completely hydrolyzed casein in excess of acid are given in Table III.

Racemization by Alkali at 25°.—The racemization experiments were carried out exactly as in the case of gelatin, except that 0.5 N, 1.0 N, and 5.0 N sodium hydroxide were used instead of 0.1 N, 1.0 N, and 3.0 N. Samples of casein equivalent to 1.000 gm. of dry substance were dissolved in 25.0 cc. of standard alkali, 1 drop of octyl alcohol being used to prevent foaming during solution.

TABLE III.

Calculation of Rotation of Mixture of Amino Acids Obtained by Hydrolysis of Casein.

Amino acid.	Per cent in casein. (1)	$[\alpha]_D^{25}$ in excess of acid. (2)	Rotation in hydrolysed casein. (3)	Racemization, racemized casein. (4)	Racemization, racemized caseose. (5)
Glycine	0.45				
Alanine	1.85	+10.4	+0.03	Complete (?).	Complete (?).
Valine	7.93	+28.8	+0.37	Partial.	Partial.
Leucine	7.92	+15.8	+0.20	"	"
Isoleucine	1.43	+40.6	+0.09		
Phenylalanine	3.88	− 7.1	−0.04	Complete.	Complete.
Tyrosine	5.70	− 8.5	−0.08	"	"
Serine	0.43	+14.4	+0.01		
Cystine	0.02	−224.0	−0.01		
Aspartic acid	4.10	+25.7	+0.17	Complete.	Complete.
Glutamic "	21.77	+34.9	+1.21	"	"
Hydroxyglutamic acid	10.50	+16.3	+0.27		
Lysine	7.72	+15.5	+0.19	Complete.	Complete.
Arginine	4.84	+21.2	+0.16	"	"
Histidine	3.39	+ 9.5	+0.05	"	"
Tryptophane	1.70	−13.5	−0.04		
Proline	8.70	−48.6	−0.68	None.	None.
Hydroxyproline	0.23	−49.2	−0.02		
Calculated rotation of completely hydrolyzed casein			+1.88		

Column 1 shows the percentages of the amino acids contained in casein as given by Gortner.[4] Column 2 shows the specific rotations of the naturally occurring amino acids, compiled from the literature. Some of these values have been changed from those recorded in the paper on gelatin.[2] Column 3 gives the calculated rotation of a mixture of amino acids corresponding to the composition of casein. This rotation is calculated for 1.000 gm. of casein in excess of acid measured in a 4 dm. tube; *i.e.*, the actual experimental conditions. Columns 4 and 5 give the results of Dakin and Dudley's[5] investigation of the racemization of casein.

[4] Gortner, R. A., in Sutermeister, E., Casein and its industrial applications, New York, 1927, 19.

[5] Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, 1913, xv, 263.

The solutions were then allowed to stand at 25° for the times indicated.

Each solution was then neutralized to litmus with hydrochloric acid. Near the neutral point a curdy, sticky solid, the racemized casein described by Dakin and Dudley,[5] separated. The solution, without removal of the solid, was evaporated to dryness in a Pyrex test-tube under reduced pressure at 50°. It was necessary to add a few drops of octyl alcohol from time to time to prevent foaming. Each residue was then treated with a small quantity of 98 per cent alcohol and again evaporated to dryness. To each residue were added 0.400 gm. of norit and 10.0 cc. of 5.0 N hydrochloric acid and the tubes were sealed. The hydrolysis for 4 hours at 125° and the subsequent operations were then carried out exactly as described under acid hydrolysis.

Control experiments for the racemizations were run by hydrolyzing for 4 hours at 125° 1.000 gm. samples of casein with 0.400 gm. of norit and 10.0 cc. of 5.0 N hydrochloric acid plus the quantities of sodium chloride equivalent to 25.0 cc. of 0.5 N, 1.0 N, and 5.0 N sodium hydroxide, respectively; *viz.*, 0.73 gm., 1.46 gm., and 7.30 gm. In the racemization experiments with 5.0 N sodium hydroxide and in the corresponding controls there was a residue of solid sodium chloride in the hydrolysis tubes.

The data are given in Table IV. The percentage racemizations calculated on the maximum observed racemization (final column) are plotted in Fig. 1.

Alkaline Hydrolysis at 25°.—The rate of hydrolysis of casein under the conditions employed in the racemizations was followed in a separate series of experiments. Samples of protein equivalent to 2.000 gm. of dry substance were dissolved in 50.0 cc. of standard alkali, 1 drop of octyl alcohol being added to each solution to prevent foaming during solution. The solutions were allowed to stand at 25°. The progress of hydrolysis was followed by the analysis of 4.00 cc. samples taken at the time intervals shown. These samples were neutralized to phenolphthalein and diluted to 25.0 cc. Total nitrogen and amino nitrogen (30 minutes) were determined as in the acid hydrolyses.

The results are given in Table V. In calculating the degree of hydrolysis from the amino nitrogen ratios, 76.2 per cent was taken as the amino nitrogen ratio of completely hydrolyzed casein

and 5.4 per cent as the amino nitrogen ratio of the original material. The percentage hydrolyses are plotted in Fig. 1.

Alkaline Hydrolysis of Gelatin at 25°.—For comparison, the hydrolysis of gelatin by sodium hydroxide at 25° was studied in a series of experiments similar to those used for casein.

TABLE IV.

Racemization of Casein by Sodium Hydroxide at 25°.

Alkali.	Time.	Total N per cc.	$\frac{\text{Amino N}}{\text{Total N}}$.	α_D^{25} corrected.	Racemization calculated on complete racemization.	Racemization calculated on maximum observed racemization.
	days	*mg.*	*per cent*	*degrees*	*per cent*	*per cent*
0.5 N	Control.	0.938	75.7	+1.82		
	1	0.903	75.1	+1.38	24	28
	2	0.882	75.3	+1.16	36	42
	3	0.861	75.0	+1.05	42	49
	7	0.759	74.3	+0.74	59	68
	10	0.762	75.6	+0.65	64	74
	15	0.757	75.4	+0.51	72	83
1.0 N	Control.	0.907	76.2	+1.85		
	1	0.878	75.9	+1.10	41	47
	2	0.665	74.0	+0.88	52	60
	3	0.829	74.5	+0.67	64	73
	7	0.860	74.3	+0.40	78	90
	10	0.762	75.0	+0.30	84	96
	15	0.801	74.5	+0.23	88	100
5.0 N	Control.	0.917	75.2	+1.80		
	1	0.826	75.1	+0.77	57	66
	2	0.841	73.8	+0.60	67	77
	3	0.868	73.9	+0.40	78	90
	7	0.718	75.7	+0.37	79	92
	10	0.889	73.3	+0.24	87	100
	15	0.834	74.6	+0.25	86	100

The results are given in Table VI. In calculating the degree of hydrolysis from the amino nitrogen ratios, 70.0 per cent was taken as the amino nitrogen ratio of completely hydrolyzed gelatin and 3.3 per cent as the amino nitrogen ratio of the original material.

Racemization of Casein by Alkali at 125°.—Samples of casein

equivalent to 1.000 gm. of dry substance were dissolved in 25.0 cc. of 1.0 N sodium hydroxide and the solutions sealed in Pyrex bomb

TABLE V.

Hydrolysis of Casein by Sodium Hydroxide at 25°.

Time.	Degree of hydrolysis.		
	0.5 N NaOH.	1.0 N NaOH.	5.0 N NaOH.
days	*per cent*	*per cent*	*per cent*
1	3.4	4.0	13.1
2	6.5	6.9	20.8
3	7.5	8.5	25.8
4	7.8	9.6	26.6
7	8.1	13.1	37.2
15	13.1	21.4	52.9
30	18.4	29.2	67.4

TABLE VI.

Hydrolysis of Gelatin by Sodium Hydroxide at 25°.

Time.	Degree of hydrolysis.		
	0.5 N NaOH.	1.0 N NaOH.	5.0 N NaOH.
days	*per cent*	*per cent*	*per cent*
1	11.2	28.6	77.4
2	20.2	41.9	83.1
4	32.1	54.9	88.6
7	38.8	63.6	92.7
10	50.0	71.4	98.7
15	52.4	77.7	100.8

TABLE VII.

Racemization of Casein by Sodium Hydroxide at 125°

Experiment No.	Amino N ratio before acid hydrolysis.	Total N per cc. after acid hydrolysis.	Amino N ratio after acid hydrolysis.	α_D^{25} corrected.
	per cent	*mg.*	*per cent*	*degrees*
1	68.4	0.668	70.1	+0.01
2	67.2	0.427	72.7	0
3	66.9	0.540	70.7	−0.03

tubes. These tubes were heated for 2 hours at 125°. The amino nitrogen ratios were determined on 4.00 cc. samples. The remain-

TABLE VIII.

Analysis of Racemized Casein.

	Weight.	C	H	P	S	N	NH_2	Moisture.	Ash.
	gm.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Non-racemized casein.		53.56	6.91	0.63	1.13	14.94	0.80	8.41	2.16
Racemized casein.									
Experiment I.......	2.74	52.03	6.58	0	0.26	12.83	0.77	8.60	2.73
Experiment II......	3.04	52.99	6.79			13.05	0.87	5.64	3.30

TABLE IX.

Hydrolysis of Racemized Casein by Hydrochloric Acid at 125°

	$\frac{\text{Amino N}}{\text{Total N}}$.	α_D^{25} corrected.
	per cent	*degrees*
Non-racemized casein. (See Table II.).........	75.8	+1.86
Corresponding racemization experiment. (See Table IV.)................................	74.5	+0.67
Racemized casein............................		
Experiment I...............................	77.2	+0.67
Experiment II..............................	76.4	+0.67

TABLE X.

Soluble Fraction from Racemized Casein.

	Before hydrolysis.		After hydrolysis.		
	Total N per cc.	$\frac{\text{Amino N}}{\text{Total N}}$.	Total N per cc.	$\frac{\text{Amino N}}{\text{Total N}}$.	α_D^{25} corrected.
	mg.	*per cent*	*mg.*	*per cent*	*degrees*
Non-racemized casein. (See Table II.).		5.4		75.8	+1.86
Corresponding racemization experiment. (See Table IV.).............		8.5		74.5	+0.67
Racemized casein....................					
Experiment I.......................	2.058	20.0	0.794	74.5	+0.61
Experiment II................. ...	1.848	24.2	0.717	73.0	+0.57

ing solutions were treated exactly as described in the racemization experiments. The final acid solutions, after hydrolysis, were optically inactive within the experimental error. The data are recorded in Table VII.

Insoluble and Soluble Fractions From Racemized Casein.[5]—Samples of casein equivalent to 5.000 gm. of dry substance were dissolved in 125.0 cc. of 1.0 N sodium hydroxide. After having stood 3 days at 25°, the solutions were made acid to litmus with glacial acetic acid. The gummy precipitates of racemized casein settled to the bottom and the supernatant liquids, containing racemized caseose and other soluble materials, were decanted.

The precipitates were washed by decantation with distilled water and dried in a vacuum desiccator. The analyses are recorded in Table VIII.

Samples of 1.000 gm. were hydrolyzed at 125° for 4 hours with 10.0 cc. of 5.0 N hydrochloric acid and 0.400 gm. of norit. The data are recorded in Table IX.

The supernatant liquids containing racemized caseose and other soluble materials were concentrated to dryness under diminished pressure at 50° and the residues were dried in a vacuum desiccator. Each residue was then dissolved in 20 cc. of 5.0 N hydrochloric acid, filtered, and diluted to 25.0 cc. with 5.0 N hydrochloric acid. Samples of 4.00 cc. of each solution were analyzed for total nitrogen and amino nitrogen. The analyses are recorded in Table X.

Samples of 10.0 cc. of each solution were sealed in Pyrex test-tubes with 0.400 gm. samples of norit and hydrolyzed at 125° for 4 hours. The solutions were filtered and diluted to 25.0 cc. and the rotations were read. Samples of 4.00 cc. were then diluted to 25.0 cc. and analyzed. The data are recorded in Table X.

SUMMARY.

1. The rotations and amino nitrogen ratios have been determined for the mixture of amino acids obtained from casein by hydrolysis with hydrochloric acid at 125°. Complete hydrolysis, together with maximum rotation, is attained when the protein is heated 4 hours with 5.0 N acid.

2. The rotations and amino nitrogen ratios have been determined for the mixture of amino acids obtained by acid hydrolysis

of casein which had previously been subjected to the action of 0.5 N, 1.0 N, or 5.0 N sodium hydroxide at 25° for different periods of time. Racemization increases with the strength of the alkali and with the time.

3. The rates of hydrolysis at 25° of casein and gelatin by 0.5 N, 1.0 N, and 5.0 N sodium hydroxide have been determined.

4. From the results of (2) and (3) it appears that casein is not composed of ketopiperazines of the type thus far studied, nor is it a simple polypeptide of the type thus far studied. The behavior of casein may be explained by assuming that the ketopiperazines contained in it are more stable than those previously studied or that the order of linkage of the amino acids is such as to facilitate racemization.

5. When casein is heated with 1.0 N sodium hydroxide at 125°, complete racemization occurs. Hence, under these conditions it is impossible to increase the rate of hydrolysis of the ketopiperazines sufficiently to avoid racemization.

6. An investigation of the soluble and insoluble fractions of racemized casein has shown that racemization precedes hydrolysis.

STUDIES ON GOSSYPOL.

IV. APOGOSSYPOL.

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In the first communication of this series (1) it was stated that gossypol dissolves in aqueous solutions of fixed alkalies, yielding a bright yellow solution. Attention was also directed to the fact that the use of more than 2 mols of alkali produces decomposition of the gossypol, which is indicated by a gradual transition in the color of the liquid from bright yellow to intense purple.

It has now been found that if hot concentrated alkali is substituted for the cold dilute reagent a different result is obtained. Under these conditions, gossypol does not at first dissolve in the alkali, but forms with it a dark orange paste. If this is frequently stirred and continuously heated, at the temperature of the steam bath, a reaction slowly takes place in which the color of the mixture becomes gray. Coincident with this color transition the viscosity of the mass greatly diminishes.

The products of the transformation are formic acid and a new phenolic substance which has been designated as *apogossypol*. Apogossypol is a colorless crystalline material having no definite melting point. It is soluble in ordinary organic solvents, and the solutions thus formed darken more or less quickly, depending upon the nature of the solvent employed. It dissolves freely in dilute alkali, from which it is precipitated by carbon dioxide. Its alkaline solutions, however, darken at once and rapidly acquire a purple color. Even crystalline apogossypol is so unstable that a few hours exposure of the dry crystals to the air and light causes

* This work was done under a research fellowship supported by the Interstate Cottonseed Crushers' Association.

them to change to a jet black powder. Another characteristic of its phenolic nature is the bluish green color reaction produced when a dilute alcoholic solution of the substance is treated with ferric chloride.

Apogossypol readily forms colorless crystalline acetyl and methoxy derivatives, which are, in contrast with the free apogossypol, quite stable. A study of these substitution products, as well as of the free phenol, has shown that apogossypol has the molecular formula $C_{28}H_{30}O_6$. Furthermore, it has been shown that

TABLE I.

Toxicity Tests Made upon Apogossypol.

The material, dissolved in cottonseed oil, was given intraperitoneally to white rats whose average weight was 185 gm.

Dose.	No. of animals used.	Deaths resulting.	Days until death occurred.	Remarks.
mg. per kg.				
50	5	0		Animals sick for 3 to 4 days after injection; appeared normal thereafter and were returned to stock after 1 mo.
60	7	2	3, 4	Slightly hemorrhagic serum in peritoneum.
75	7	4	1, 4, 5, 5	Animals which survived did not regain normal condition; appeared emaciated; hair was rough; lost weight. One died after 6 wks.
90	2	2	$1\frac{1}{2}$, $1\frac{1}{2}$	
100	2	2	$\frac{1}{2}$, 1	

there are six acetyl groups in the acyl derivative. No direct determination of the methoxy groups in the methyl ether could be made, since the substance was completely inert in boiling hydriodic acid under the conditions used in the Zeisel method. Carbon and hydrogen figures, however, checked very well for a hexamethyl ether. It therefore follows that all the oxygen atoms in apogossypol are present as hydroxyl groups. From a consideration of the molecular formula for gossypol, $C_{30}H_{30}O_8$, it will be seen that strong hot alkali, under the conditions of the experiment, functions only in removing the two carbonyl groups from the gossypol molecule. Since formic acid is the only other sub-

stance produced by the reaction, the carbonyl groups must have been eliminated as formic acid. Such a process requires that each mol of gossypol should produce 1 mol of apogossypol and 2 mols of formic acid. The yield of each of these substances actually obtained corresponded to 97.5 per cent and 95.3 per cent respectively of that demanded by the theory.

It is noteworthy that in the elimination of the two carbonyl groups from gossypol the characteristic yellow color of the substance disappears. This substantiates the suggestion previously made (1) that the chromophores of gossypol are carbonyl groups.

Finally, the toxicity of apogossypol was compared with that of gossypol. Freshly prepared material dissolved in cottonseed oil was given intraperitoneally to white rats in the manner previously adopted in the toxicity work with gossypol. The results are recorded in Table I. When these data are compared with those previously recorded for gossypol (1, 2), it will be seen that apogossypol is the less toxic of the two. The lethal dose of apogossypol when given as indicated is from 60 to 75 mg. per kilo of body weight. Its physiological action, however, is in sharp contrast with that of gossypol as no chronic toxic effects follow the administration of small doses. Its action seems to be restricted to the development of acute toxic symptoms only.

EXPERIMENTAL.

Preparation of Apogossypol.

5 gm. of gossypol and 45 cc. of a 40 per cent solution of sodium hydroxide, contained in a large test-tube, were frequently stirred and heated in a steam bath for 30 minutes. At first the gossypol did not dissolve, but formed a dark orange-colored paste. As the reaction proceeded the consistency of the mass became thinner until at its termination almost all the suspended material had dissolved. This transformation was accompanied by a color reaction in which the dark orange at first produced changed to yellow, then to gray, and finally the terminal liquid phase was decidedly dark. At the end of the time indicated the process was completed, and the reaction mixture was removed from the heat and cooled. The test-tube was filled with crushed ice, and concentrated sulfuric acid was added with stirring until an acid

reaction to Congo red was obtained. The white precipitate formed was filtered upon a Buchner funnel and washed with water. The product was then dissolved from the filter with ether, the excess of water was removed by a separatory funnel, and the ethereal solution was dried with anhydrous sodium sulfate. The liquid was then filtered, the ether was evaporated on a steam bath, and the residue was dissolved in 25 cc. of hot methanol. Water was added to the resulting solution until it became slightly turbid. Upon scratching the flask, apogossypol crystallized at once. The yield was 4.35 gm., or 97.5 per cent of the theory. Generally it had a light brown appearance, but if small quantities of gossypol were worked up and the final steps were carried out rapidly the apogossypol was at first entirely colorless. This material had the properties which were mentioned before. When quickly dried in a high vacuum over both sulfuric acid and potassium hydroxide with the exclusion of light the following results were obtained for carbon and hydrogen.

96.9 mg. substance gave 255.1 mg. CO_2 and 57.1 mg. H_2O.

For $C_{28}H_{30}O_6$.	Calculated.	C 72.70 per cent	H 6.54 per cent.
	Found.	" 71.82 " "	" 6.59 " "

From the preparative standpoint a better procedure than that just given for obtaining the apogossypol from the reaction mixture consists in extracting the precipitate from the acidified mixture directly with ether. The other steps are the same as those in the method just recorded but in this way a tedious filtration is avoided.

Examination of Acid Filtrate.

The acid filtrate from the apogossypol was distilled in order to examine the distillate for volatile fatty acids. The total quantity of acid obtained required 184 cc. of 0.1 N sodium hydroxide for neutralization. The solution of sodium salts thus obtained was evaporated to dryness on a steam bath, the residue was dissolved in approximately 100 cc. of water, and slightly more than enough sulfuric acid to liberate the organic acid was added. The solution was then diluted to 150 cc. and distilled at a constant volume as directed by Dyer (3) to obtain a distillation curve of the acid.

The curve obtained was a straight line coinciding almost exactly with the distillation curve of formic acid. The information thus obtained indicated that the substance being dealt with was entirely formic acid. As confirmatory evidence the tests for formic acid recommended by Mulliken (4), *i.e.* the reduction of mercuric oxide to mercury and the decomposition of formic acid by sulfuric acid with the evolution of carbon monoxide and its subsequent identification, were positive. Also mercuric chloride was reduced to mercurous chloride, silver nitrate was reduced to silver, and potassium permanganate was reduced in the cold. These tests were made upon the sodium salt obtained by evaporating the neutralized distillate from the running of the distillation curve. 184 cc. of 0.1 N formic acid are equivalent to 0.846 gm. of the acid. This corresponds to a yield of 95.3 per cent of the theory on the basis of 2 mols of formic acid from 1 mol of gossypol.

Apogossypol Hexaacetate.

1 gm. of apogossypol dissolved in 5 cc. of pyridine was acylated with 2 cc. of acetic anhydride. The solution was heated to its boiling point and then allowed to cool and stand at the temperature of the room for 1 hour. When 50 cc. of water were added to the reaction mixture the acetyl derivative was precipitated as a cream-colored mass, which soon crystallized. Another procedure which was used as a preparative method consisted in following the directions for the preparation of apogossypol given before to the point where the phenol was dissolved in methanol. Instead of the residue being dissolved in this solvent, it was taken up in pyridine and treated directly with acetic anhydride, the proportions of reagents and method just given being used. In this way much time was saved, and equally good results were obtained. The yield of the crude product was uniformly 90 per cent of the theory.

The substance was purified by dissolving 2 gm. in 75 cc. of boiling ethyl acetate and adding an equal volume of petroleum ether. Crystallization began at once and was complete within an hour. The yield was 1.8 gm., or 90 per cent. It consisted of large colorless rods which began to soften at 285° and melted at

291° (corrected). A molecular weight determination made by the Rast camphor method (5) gave the following results:

10.4 mg. substance dissolved in 100.2 mg. camphor, $\Delta = 5.6°$.

$$\text{Mol. wt.} = \frac{(40,000)\ (10.4)}{(100.2)\ (5.6)} = 743.$$

Acetyl was determined by Perkin's method for both O-acyl and N-acyl compounds (6).

I. 0.3990 gm. substance required 32.0 cc. 0.1 N alkali (N-acyl method).
II. 0.4205 " " " 24.96 " 0.1 " " (O-acyl ").
I. 83.4 mg. substance gave 205.6 mg. CO_2 and 44.5 mg. H_2O.
II. 83.8 " " " 206.3 " " " 44.9 " "
For $C_{40}H_{42}O_{12}$. Calculated. Mol. wt. 714.5, acetyl (six CH_3CO groups) 36.13 per cent, C 67.21 per cent, H 5.93 per cent.
Found. I. Mol. wt. 743, acetyl 34.5 per cent, C 67.25 per cent, H 5.97 per cent.
II. Acetyl 25.54 per cent, C 67.16 per cent, H 6.00 per cent.

It will be seen from the results recorded for acetyl that in the substance under consideration, as in hexaacetyl gossypol, two of the acetyl groups are more resistant to the action of hydrolytic agents than the remaining four. The acetyl value obtained with the O-acyl method is in good agreement with that required if only four acetyl groups were removed. Under this condition the theory requires 24.09 per cent acetyl.

The optical properties of the substance were determined by Mr. George L. Keenan of the Food, Drug and Insecticide Administration of the Department of Agriculture, who reported as follows: "The material crystallizes in rods and plates. Its indices of refraction are $n_\alpha = 1.525$, $n_\gamma = 1.680$. Birefringence is extremely strong, elongation is negative, and the extinction is parallel."

Apogossypol Hexamethyl Ether.

5 gm. of gossypol were converted into apogossypol by treatment for 30 minutes in a steam bath with 45 cc. of 40 per cent sodium hydroxide as previously outlined. The resulting solution was cooled and diluted with an equal volume of water, after which 15 cc. of dimethyl sulfate were added, and the mixture was

vigorously stirred until the reagent was consumed. This was indicated when the methylated product acquired a hard lumpy consistency. After the mixture was cooled somewhat another 15 cc. of methyl sulfate were added and the process was repeated. This completed the reaction. The alkaline liquid was diluted with water, filtered, and thoroughly washed. The dried product which weighed 4.5 gm. was digested with 25 cc. of warm methanol and the insoluble material which weighed 3.5 gm., was removed by filtration. This substance melted at between 230–240°. It was dissolved in boiling benzene, the solution was decolorized with norit, and the methyl ether was crystallized by adding 4 volumes of methanol. The product thus purified consisted of colorless spindle-shaped crystals which melted at 259° (corrected). Its solution was optically inactive as was that of the gossypol from which it was prepared. A molecular weight determination made by the Rast method gave the following results.

11.0 mg. substance dissolved in 106 mg. camphor, $\Delta = 7.5°$.

$$\text{Mol. wt.} = \frac{(40{,}000)\ (11)}{(106)\ (7.5)} = 553.$$

I. 94.0 mg. substance gave 257.8 mg. CO_2 and 64.9 mg. H_2O.
II. 90.3 " " " 247.6 " " " 62.4 " "

For $C_{34}H_{42}O_6$. Calculated. Mol. wt. 546.5, C 74.69 per cent, H 7.75 per cent.
Found. I. Mol. wt. 553.0, C 74.82 per cent, H 7.73 per cent.
II. " 74.80 " " " 7.73 " "

Methoxy determinations were not made, since boiling hydriodic acid as used in the Zeisel method had no effect upon the material.

Mr. Keenan reported the following data concerning the optical properties of the compound. "Apogossypol hexamethyl ether consists of spindle-shaped crystals whose indices of refraction are $n_\alpha = 1.600$, $n_\gamma = 1.675$. The birefringence is extremely strong and the polarization colors are brilliant, the bands representing the successive orders being unusually distinct."

SUMMARY.

1. Gossypol is converted into formic acid and apogossypol, a new phenolic substance, when it is treated with 40 per cent sodium hydroxide at the temperature of the steam bath for ½ hour.

2. These substances are produced in the proportion of 2 mols of formic acid to 1 mol of apogossypol.

3. Apogossypol has the molecular formula $C_{28}H_{30}O_6$. It is formed by the elimination of the two carbonyl groups of gossypol as formic acid.

4. All the 6 oxygen atoms of apogossypol are present as hydroxyl groups. The phenol accordingly forms a hexaacetyl derivative and a hexamethyl ether by the replacement of the hydroxyl hydrogens by acetyl and methyl radicals. These compounds have been described. In the hexaacetate, as in hexaacetyl gossypol, two of the acetyl groups are more resistant to hydrolytic agents than the remaining four. The direct determination of the methoxy groups in the hexamethyl ether could not be made as the material was entirely inert in boiling hydriodic acid as used in the Zeisel method.

5. The toxicity of apogossypol was determined and compared with that of gossypol. Of the two, apogossypol was considerably less toxic. The lethal dose, given intraperitoneally to white rats, was found to be from 60 to 75 mg. per kilo of body weight. Apogossypol differs from gossypol in its physiological action in causing acute toxic effects only.

BIBLIOGRAPHY.

1. Clark, E. P., *J. Biol. Chem.*, 1927, lxxv, 725.
2. Clark, E. P., *J. Biol. Chem.*, 1928, lxxvi, 229.
3. Dyer, D. C., *J. Biol. Chem.*, 1916–17, xxviii, 445.
4. Mulliken, S. P., A method for the identification of pure organic compounds, New York, 1905, i, 83.
5. Rast, K., *Ber. chem. Ges.*, 1922, lv, 1051.
6. Perkin, A. G., *J. Chem. Soc.*, 1905, lxxxvii, 107.

A STUDY ON THE EFFECT OF FEEDING CREATINE ON GROWTH AND ITS DISTRIBUTION IN THE LIVER AND MUSCLE OF NORMAL MICE.

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There is no extensive literature relative to the storage of creatine in the tissues of the organism after the administration of creatine and of related compounds. An increase in the creatine content of muscle after subcutaneous injections has been noted by Myers and Fine (1) and by Folin and Denis (2, 3) after intestinal and intravenous injections of creatine. Myers and Fine (4) found a very slight increase in the creatine content of muscles of rats fed on a protein rich in arginine. Chanutin (5) has reported that after feeding rats on an adequate diet containing 2.6 per cent of creatine, the liver was the only tissue in which a significant rise in the creatine concentration was noted. The increase in the creatine concentration of the muscles of these animals was, however, very slight.

The idea has been advanced that creatine might serve as a food. Benedict and Osterberg (6) demonstrated that creatine was partially retained and utilized by dogs and that these animals showed an increase in weight and a positive nitrogen balance. It was shown by Chanutin (7) that a positive nitrogen balance follows creatine feeding in man. Chanutin (5) has presented evidence that the feeding of adequate diets containing creatine to young rats for a period of 2 months has no effect on the growth curve.

The present investigation was undertaken in view of the possibility that a large creatine concentration in the diet of white

* This work was done in Dr. V. C. Myers' laboratories while the senior author was on leave of absence from the University of Virginia.

mice might produce pronounced results in the growth curve and in the storage of this material in the liver and muscles.

EXPERIMENTAL.

The white mouse served as the experimental subject. The diets used are shown in Tables I and II. They are based on the observations of Wheeler (8) and Beard (9) that a concentration of at least 31 per cent of casein is required by mice for satisfactory growth. Cod liver oil and dried yeast were incorporated in the diet to supply vitamins A and B. The creatine used in the experiments was prepared by double recrystallization of a crude commercial product. Analyses of this product gave satisfactory results. The stock food mixtures were made at frequent intervals and kept in a cold room.

In order to obtain the liver and muscles for the analysis of creatine the mice were killed at definite periods by a blow on the head. The animals were immediately decapitated and the tissues mentioned above were dissected free and cut into small pieces at once with scissors. The tissues were immediately transferred to stoppered containers and weighed. The analysis for creatine (total creatinine) was made by the method of Rose, Helmer, and Chanutin (10). It should be emphasized that the tissues to be analyzed were treated with sulfuric acid within 10 minutes after the death of the animal. The assumption is made that the liver and muscles contain very small amounts of creatinine.

Effect of Creatine upon Growth of Mice.

The mouse is particularly well adapted to growth experiments since it reaches full weight and maturity in a few months. Young male mice, isolated in individual cages, were placed on the diets given in Table I for a period of 8 weeks. The data obtained indicate that the growth curves of white mice are not influenced by the addition of creatine to adequate diets.

Effect of High Protein Diets upon Creatine Content of Liver and Muscle.

The theory that arginine is the mother substance of creatine has long been a matter of discussion. But no conclusive evidence

has thus far been presented which proves that creatine originates from arginine. In order to study this question mice were placed on high edestin and casein diets. The former protein yields 14.1 per cent arginine and the latter 3.8 per cent (11). A control group on a normal diet was also studied. After a period of about

TABLE I.

Composition of Diets.

Diet No.	1	2	3	4
	gm.	*gm.*	*gm.*	*gm.*
Casein	31	31	31	31
Starch	38	36	33	28
Crisco	18	18	18	18
Cod liver oil	3	3	3	3
Dried yeast	3	3	3	3
Salt mixture*	7	7	7	7
Creatine		2	5	10
Total	100	100	100	100

* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

TABLE II.

Composition of Diets.

Diet No.	5	6
	gm.	*gm.*
Casein	75	
Edestin		75
Starch	12	12
Salt mixture*	7	7
Cod liver oil	3	3
Yeast	3	3
Total	100	100

* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

3 weeks on the above diets, the liver and muscle creatine was determined.

The results of the experiment on mature mice are shown in Table III. The analytical data for the creatine of the liver in the various groups are quite uniform. The dietary differences have

TABLE III.

Creatine Content of Liver and Muscle As Influenced by High Protein Feeding (Female).

Mouse No.	Duration of experiment.	Weight at end of experiment.	Liver creatine.		Muscle creatine.
Diet 1 (control).					
	days	*gm.*	*mg.*	*per cent*	*per cent*
51	24	24.0	0.40	0.042	0.375
52	24	26.0	0.43	0.036	
53	24	25.0	0.40	0.033	0.358
54	24	24.5	0.40	0.044	0.342
55	25	25.0	0.46	0.037	0.327
56	25	32.0	0.48	0.029	0.365
57	25	29.0	0.48	0.029	0.333
58	27	29.0	0.52	0.033	0.396
59	27	24.0	0.56	0.041	0.392
60	27	28.0	0.38	0.036	0.386
Average...			0.45	0.036	0.364
Diet 5 (high casein).					
61	24	25.0	0.59	0.033	0.353
62	24	27.0	0.49	0.035	0.319
63	24	20.0	0.45	0.036	0.355
64	24	27.0	0.47	0.034	0.359
66	25	33.0	0.39	0.025	0.355
67	25	28.0	0.46	0.029	0.348
68	25	25.0	0.43	0.032	0.319
Average...			0.47	0.032	0.344
Diet 6 (high edestin).					
69	24	26.0	0.45	0.032	0.349
70	24	20.0	0.37	0.033	0.371
71	24	24.0	0.41	0.037	0.331
73	25	24.0	0.38	0.028	0.329
74	25	27.0	0.48	0.033	0.333
75	25	26.0	0.41	0.028	0.304
76	25	22.0	0.35	0.029	0.315
Average...			0.40	0.031	0.333

no effect on the creatine concentration of the liver. There is noted, however, a marked difference in the muscle creatine concentration in the mice on the different diets. The averages obtained for the respective groups seem to be representative, despite the irregularity of muscle creatine in individual mice. It is believed that the period of feeding was long enough to yield convincing results. The only possible criticism which might be raised is that the diet was so high in protein as to be unbalanced. On the high edestin diet the creatine concentration of the muscle is lowest, showing an average decrease of 9 per cent from the normal. The muscle of the high casein-fed mice also showed a decrease in creatine concentration but not to the same extent as noted above.

Effect of Creatine Feeding upon Creatine Concentration of Liver and Muscle of the Growing Mouse.

Young mice about 1 month old were placed on adequate diets containing various percentages of creatine. These animals were divided into two groups and analyses on liver and muscle were made after about 20 and 58 days of creatine ingestion. Through these periodic analyses it was thought that it would be possible to determine whether or not time is a factor in creatine storage. Furthermore, the concentration of the creatine in the present diets was much higher than in the diets which had been previously fed to experimental subjects over a relatively prolonged period.

The data on the influence of creatine ingestion on the creatine concentration of the liver and muscle are summarized in Tables IV and V. After feeding mice for about 20 days on diets containing 2 and 5 per cent creatine (Table IV) it is noted that the muscle creatine is increased approximately 7 and 12 per cent, respectively, above the normal concentration. These increases are undoubtedly indicative of a storage of excess creatine in muscle. The liver creatine in this same group of animals is very markedly increased but wide variations in creatine determinations for individual animals make it impossible to average the data so that reliable comparisons can be made. It is difficult to explain the variations in the creatine concentration of the liver. The fairly constant results obtained for the livers of the control animals would indicate a reliable technique in analysis.

TABLE IV.

*Effect of Creatine Feeding for a Relatively Short Period upon Creatine Concentration of Liver and Muscle of the Growing Mouse.**

Mouse No.	Weight at end of experiment.	Liver creatine.		Muscle creatine.
Diet 1 (control).				
	gm.	*mg.*	*per cent*	*per cent*
78	18.0	0.28	0.030	0.360
79	20.0	0.25	0.023	0.362
80	14.5			0.360
81	17.0	0.53	0.048	0.376
82	18.0	0.29	0.028	0.341
83	16.0	0.31	0.032	0.399
84	18.5	0.33	0.032	0.369
Average....		0.33	0.032	0.367
Diet 2 (2 per cent creatine).				
92	22.5	0.90	0.055	0.402
93	27.0	4.48	0.271	0.413
94	23.0	4.78	0.299	0.362
95	19.0	1.24	0.114	0.397
96	17.0	0.87	0.106	0.408
97	21.0	0.47	0.050	0.376
98	14.5	0.43	0.046	0.400
Average....		1.88	0.134	0.394
Diet 3 (5 per cent creatine).				
102	21.0	1.91	0.133	0.434
103	22.0	1.84	0.147	0.375
104	24.0	2.09	0.198	
105	20.0	2.16	0.217	0.433
106	21.0	2.30	0.175	0.350
107	18.0	1.07	0.089	0.428
108	17.0	0.58	0.051	0.418
109	17.0	0.84	0.071	0.414
110	15.0	0.49	0.061	0.451
Average....		1.47	0.127	0.413

* Duration of experiment about 3 weeks (19 to 21 days).

TABLE V.

*Effect of Creatine Feeding for a Relatively Long Period upon Creatine Content of Liver and Muscle of the Growing Mouse.**

Mouse No.	Liver creatine.		Muscle creatine.
		Diet 1 (control).	
	mg.	*per cent*	*per cent*
24	0.53	0.034	0.378
25	0.46	0.024	0.379
26	0.46	0.028	0.378
27	0.44	0.035	0.365
Average.........	0.47	0.030	0.375
		Diet 2 (2 per cent creatine).	
29	0.94	0.059	0.372
30	0.55	0.032	0.415
31	1.01	0.074	0.376
32	2.00	0.128	
Average.........	1.12	0.073	0.387
		Diet 3 (5 per cent creatine).	
33	3.72	0.194	0.396
34	2.62	0.232	0.395
35	0.90	0.071	0.412
36	3.80	0.202	
Average.........	2.76	0.175	0.401
		Diet 4 (10 per cent creatine).	
37	1.49	0.089	0.416
38	4.37	0.231	0.398
39	2.00	0.124	0.397
40			0.387
41	4.93	0.273	0.386
Average.........	3.20	0.179	0.397

* Duration of experiment about 8 weeks.

Unfortunately, insufficient experimental data on mice fed for a long period (Table V) make it impossible to study the relationships of creatine storage to the length of time of feeding. It is

certain, however, that the capacity of the muscle to store creatine cannot be increased above the level obtained when creatine was fed for a shorter period, even though a larger amount of creatine was ingested (10 per cent) by one group of animals in this series.

Variations in the liver creatine in these experiments make comparisons impossible. Nevertheless, there seems to be every indication that this tissue also has a saturation limit.

TABLE VI.

Effect of Creatine Feeding for a Short Period (9 Days) upon Creatine Concentration of Liver and Muscle of the Mature Mouse (Male).

Mouse No.	Weight at end of experiment.	Liver creatine.		Muscle creatine.
Diet 1 (control).				
	gm.	*mg.*	*per cent*	*per cent*
113	21.0	0.48	0.032	0.379
114	20.0	0.49	0.035	0.342
115	20.0	0.44	0.037	0.383
116	22.0	0.37	0.031	0.375
117	17.0			0.365
Average....		0.44	0.034	0.369
Diet 3 (5 per cent creatine).				
119	24.5	0.57	0.049	0.423
120	17.0	0.41	0.046	0.402
121	21.0			0.400
122	18.5	0.43	0.043	0.404
123	21.0	0.46	0.042	0.423
124	20.0	0.63	0.066	0.399
Average....		0.50	0.049	0.408

Effect of Creatine Feeding upon Creatine Concentration of Liver and Muscle of the Mature Mouse.

Tables VI and VII contain data upon the creatine concentration of liver and muscle of mature mice on normal and creatine diets for relatively short and long periods. It will be noted that the muscle of mice fed a diet containing 5 per cent creatine for 9 days seems to have reached a saturation capacity. Feeding of diets

containing 5 and 10 per cent creatine for 35 days has no further influence on creatine storage in the muscle.

The data obtained for the liver present interesting results. The liver of animals fed for 9 days shows a comparatively small increase in creatine concentration. The analyses obtained in this experiment are rather constant in contrast to the large

TABLE VII.

Effect of Creatine Feeding for a Long Period (35 Days) upon Creatine Concentration of Liver and Muscle of the Mature Mouse (Female).

Mouse No.	Weight at end of experiment.	Liver creatine.		Muscle creatine.
Diet 1 (control).				
	gm.	*mg.*	*per cent*	*per cent*
42	23.5	0.42	0.031	0.360
43	23.0	0.46	0.034	0.357
44	24.0	0.40	0.030	0.359
Average....		0.43	0.032	0.359
Diet 3 (5 per cent creatine).				
45	30.0	6.62	0.387	0.420
46	27.0	5.90	0.378	0.418
47	24.0	6.37	0.409	0.451
Average....		6.29	0.391	0.429
Diet 4 (10 per cent creatine).				
48	25.0	2.66	0.247	0.390
49	21.0	2.41	0.206	0.411
50	18.0	1.37	0.165	0.392
Average....		2.15	0.206	0.397

variations noted when there are marked increases as in the case of mice fed for longer periods.

Effect upon Liver and Muscle Creatine after Discontinuing Creatine Diet.

After placing mature mice on creatine diets for a sufficient length of time to assure maximum storage of creatine in the liver

and muscle, a normal diet was substituted in order to study the effect of sudden withdrawal of creatine. The results (Table VIII) indicate a rapid withdrawal of creatine from the liver during the 3 day ingestion of the normal diet. In two animals studied the creatine concentration dropped slightly below normal. The muscle, on the other hand, shows practically no loss of excess creatine under the experimental conditions.

TABLE VIII.

Effect of Discontinuing Creatine Diet upon Creatine Concentration of Liver and Muscle of the Mature Mouse.

Mouse No.	Weight at end of experiment.	Liver creatine.		Muscle creatine.
Diet 3 (5 per cent creatine) fed for 16 days.				
	gm.	*mg.*	*per cent*	*per cent*
125	32.0	5.35	0.225	0.395
126	31.0	3.08	0.151	0.398
127	25.0	3.77	0.270	0.377
128	22.0	3.54	0.247	0.452
Fed creatine diet for 13 days; placed on Diet 1 (creatine-free) for 3 days.				
133	26.5	1.50	0.087	0.407
134	33.5	0.70	0.041	0.380
135	28.0	0.99	0.063	0.395
Diet 4 (10 per cent creatine) fed for 16 days.				
129	31.0	5.80	0.297	0.409
130	23.5	6.72	0.449	0.436
Fed creatine diet for 13 days; placed on Diet 1 for 3 days.				
131	26.0	0.35	0.029	0.416
132	28.5	0.46	0.026	0.383

DISCUSSION.

A discussion of the fate of ingested creatine must necessarily be hypothetical, since no definite explanation of its metabolism has been satisfactorily advanced. It has been generally assumed that ingested creatine is either deposited in the tissues, utilized by the organism, or partly excreted by the kidney. Reference has

been made above to experiments which demonstrated that creatine can be stored by the muscles after parenteral administration. There is, however, no evidence available to show an appreciable storage of creatine in the tissues after its ingestion. Metabolism studies indicate a retention of this material but nothing is known concerning its fate in the organism.

The data presented in this paper are of interest from several points of view. It has been shown that voluntary muscle can store ingested creatine in appreciable amounts. This is not in accord with the evidence presented by one of us (5) with the rat as the experimental subject. In this former investigation it was shown that the creatine content of muscle of rats fed for about 2 months on an adequate diet containing 2.6 per cent creatine was not affected. It is difficult to reconcile these facts except on the basis of a difference in the method of quantitative handling of creatine in any given species. In this connection it should be noted that the average creatine concentration of the muscles of the white rat is 0.449 per cent in contrast to 0.367 per cent in the mouse. It is probable that the higher creatine concentration in the muscle of the rat may be in some way responsible for the failure of these animals to elaborate an additional storage of creatine. Mice fed in a similar manner over a comparatively short period showed a distinct storage of muscle creatine. It would seem from the experimental data that the muscle has a limited storage capacity for creatine since continued ingestion of large or small quantities of creatine over various lengths of time has no effect on the ability of the muscle to store this material beyond a certain concentration. On the basis that the muscles of all the creatine-fed animals in these experiments have reached a saturation point, a rough estimation of the ability of the muscle to store excess creatine was made. The average creatine concentration in the muscle of 28 normal mice is 0.367 per cent; the average obtained for 38 creatine-fed animals is 0.403 per cent. This represents an average increase of 9.8 per cent in the concentration of creatine in the muscle of creatine-fed mice. Under the experimental conditions given, *the conclusion appears to be warranted that the ability of muscle to store creatine is limited.*

No relationship was observed between the arginine content of the diet and the creatine content of muscle in these experiments.

A review of the literature (12) discloses many conflicting ideas concerning the relationship of arginine to creatine-creatinine metabolism. It is difficult to account for the deviation below the normal noted in the concentration of muscle creatine of mice on high protein diets, especially in the case of edestin. The experimental animals were in good condition and apparently ate an adequate amount of food.

The experimental evidence concerning the rôle of the liver in creatine-creatinine metabolism is limited and is in no way convincing. This question and the literature pertaining to it have been discussed in a former communication (5). Mann and McGath (13) have presented evidence to show that removal of the liver apparently does not modify in any respect the formation of creatine. As a result of data obtained on the creatine content of the livers of creatine-fed rats, Chanutin (5) pointed out a possible relationship between the liver and creatine metabolism. The liver was the only organ in which a significant increase in creatine concentration was noted in rats fed on creatine-containing diets.

The ability of the liver to store relatively large quantities of creatine has again been demonstrated in these experiments. Inasmuch as there seems to be no relationship whatsoever between the amount of creatine stored and the amount ingested, it is impossible to discuss the significance of large and small increases in the liver creatine.

There are several possibilities to explain the increase in creatine concentration of the liver. It may be assumed that creatine is stored temporarily in this organ in large quantities because of the relationship of the liver to the intestinal circulation. Evidence in favor of this idea is shown in the rapid disappearance of creatine from the liver when the experimental animals were placed on a normal diet. On the other hand, mice fed creatine diets for a short period of time gave results which would appear to contradict the idea that liver served only as a temporary storehouse. The muscle creatine of these animals became "saturated," while the liver creatine was increased only slightly above normal. If it were entirely a question of storage it is logical to assume that the liver would certainly hold larger amounts of creatine under these conditions.

There are no available data to offer at present concerning the relationship of the liver to creatine-creatinine metabolism. The view of many workers (discussed by Hunter (12)) that a derangement of carbohydrate metabolism, which at the same time would involve the liver, contributes to a change in creatine metabolism seems to be attractive. Nevertheless, it is important to emphasize the fact that no evidence has been given to demonstrate the possible rôle the liver itself might play. Our results might lead one to infer that the liver may be concerned in creatine-creatinine metabolism. With our experimental methods, it has not thus far been possible to determine specifically any probable function of the liver in this connection. Inasmuch as the liver seems to show definite changes in its creatine content under various dietary conditions and in animals other than the mouse, the possibility of a relationship between the liver and creatine-creatinine metabolism has been suggested.

SUMMARY.

The feeding of adequate diets containing creatine (2, 5, and 10 per cent) to young mice for a period of 2 months has no effect on the growth curve.

The average creatine concentrations for the muscle and liver of the white mouse are 0.367 and 0.035 per cent, respectively.

The feeding of an arginine-rich protein does not increase the creatine concentration of the liver and muscle.

The administration of creatine to mice was found to induce an accumulation of creatine in the liver and muscle. The storage produced by creatine feeding in the muscle reached a maximum regardless of the amount of creatine in the diet and the duration of the experiment. The accumulation of creatine in the liver was not as rapid and a marked irregularity in the amount stored was noted. The withdrawal of creatine from the diet and subsequent feeding of a normal diet for a short period results in no perceptible change in the excess creatine stored in the muscle, while the concentration of creatine in the liver was markedly diminished under these conditions.

BIBLIOGRAPHY.

1. Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1913-14, xvi, 169.
2. Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 153.
3. Folin, O., and Denis, W., *J. Biol. Chem.*, 1914, xvii, 493.
4. Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1915, xxi, 389.
5. Chanutin, A., *J. Biol. Chem.*, 1927, lxxv, 549.
6. Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1923, lvi, 229.
7. Chanutin, A., *J. Biol. Chem.*, 1926, lxvii, 29.
8. Wheeler, R., *J. Exp. Zool.*, 1913, xv, 209.
9. Beard, H. H., *Am. J. Physiol.*, 1925-26, lxxv, 645.
10. Rose, W. C., Helmer, O. M., and Chanutin, A., *J. Biol. Chem.*, 1927, lxxv, 543.
11. Mendel, L. B., Nutrition: The chemistry of life, New Haven, 1923, 115.
12. Hunter, A., *Physiol. Rev.*, 1922, ii, 586.
13. Mann, F. C., and McGath, T. B., *Ergebn. Physiol., 1. Abt.*, 1924, xxiii, 212.

RICKETS IN RATS.

IV. THE EFFECT OF VARYING THE ACID-BASE CONTENT OF THE DIET.*

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Alterations in the acid-base value of the diet are believed to affect directly the cause and cure of rickets and tetany, and the absorption and excretion of calcium and phosphorus.

Zucker (1) has offered evidence that if the acidity is lessened, a rickets-producing diet fails to produce rickets. McClendon (2) has stated that the addition of alkali to a diet increases its power to produce rickets. Jones (3) has reported the cure of rickets by the addition of acid to the diet of ricketic children, and recently (4) has emphasized the acid-base factor in the cause and prevention of rickets in dogs. McCollum *et al.* (5) believe that the acid-base factor is unimportant. They found that the same degree of rickets resulted from his diet whether it contained $CaCO_3$ or $CaCl_2$ in an equal amount. Mellanby (6) also regards the acid-base factor as unimportant in the causation of rickets. On the other hand, Shelling (7) has found that acid diets produce a more severe degree of rickets than do alkaline diets.

Tetany is known to be related to the phosphates and also to acid and alkali. Binger (8) found that alkaline phosphates given intravenously produced tetany; acid phosphates did not produce tetany though the serum calcium was low. Salvesen, Hastings, and McIntosh (9) produced tetany by feeding phosphates—acid and alkaline. The present accepted cure of tetany is to feed acid or acid-producing substances such as $CaCl_2$ or NH_4Cl (10, 11).

Rats made ricketic with a ration high in calcium, low in phosphorus, and lacking vitamin D, were cured by the addition of phosphate to the diet (12, 13). The salt added was monobasic

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or acid sodium phosphate. How far had the altered acid-base relationship been the cause of healing? Coincident with the healing of rickets, the rats showed tetany (12). Some died in convulsions, some showed carpopedal spasm, and all had low values for calcium in the blood serum. It was our purpose, therefore, to investigate the effect upon the healing of rickets and tetany, of varying amounts of acid and base in the diet.

Plan of Experiment.—Sufficient phosphate was added to the rickets-producing diet to change the ratio of Ca:P from 4.25 to 0.95. The additions were made with Na_3PO_4, NaH_2PO_4, H_3PO_4, and H_3PO_4 plus HCl. The amount of phosphorus was the same in all cases but the resulting acid-base values varied widely. Rats were prepared for the experiment as in the previous studies and the analytical procedure was the same (12, 14, 15). Rats 28 days old, reared on Sherman's "normal" diet (Diet B) (16), were made ricketic by Steenbock's Diet 2965 (17) plus 10 per cent lard, which was given for 21 days. Data were obtained in regard to: (1) blood serum analyses for calcium and phosphorus, (2) histological examination of the bones, (3) bone analyses, and (4) metabolism of calcium and phosphorus.

Calculation of Diets.—Sherman's Diet B is a neutral diet. 100 gm. of the food are equivalent to 13 cc. of 0.1 N alkali. This value was calculated according to the procedure of Sherman and Gettler (18). They attributed to PO_4, however, a valence of 2.0. We have calculated this as 1.8, which is its neutralizing value at the reaction of the body. The diet (No. 2965) devised by Steenbock and Black is slightly acid without the $CaCO_3$, but with this salt addition equals 530 cc. of 0.1 N alkali per 100 gm. The diet was modified by the addition of 0.850 gm. of P as Na_3PO_4, NaH_2PO_4, or H_2PO_4. The alkalinity was 859, 311, and 37 cc. of 0.1 N alkali respectively. To make the diet acid, 350 cc. of 0.1 N HCl were added to the last diet, so that it equalled 313 cc. of 0.1 N acid.

Conduct of Experiment.

Experiments were made in July, 1926, and July and August, 1927. During the production of rickets, as previously reported (12), the animals grew a gm. a day and were quiet and docile. When the phosphate of different degrees of acidity was added, the dif-

ferent groups behaved quite differently. In the group receiving Na_3PO_4 no fatalities occurred. The animals all showed muscular spasm. In the rats receiving H_3PO_4 + HCl no convulsions were observed and all lived. In the group receiving sufficient H_3PO_4 to make the diet practically neutral all had convulsions and all died.

To obtain living animals, fed upon the neutral diet, the procedure was modified so that the diet was shifted by stages. On the 1st day one-fourth of the H_3PO_4 diet and three-fourths of the rickets-producing diet were given; on the 2nd day one-half and one-half respectively; on the 3rd day one-fourth and three-fourths; and on the 4th day the H_3PO_4 diet was given and the metabolism study begun. Because of this gradual change, the metabolism periods, therefore, are not strictly comparable to those of the other groups; but the change was made in such a way that all the rats were of the same age at the time when the metabolism studies were begun. In this group no convulsions occurred.

Results.

1. Blood Serum Analyses.—The values for the blood serum analyses at the end of 2 weeks are shown in Table I. All cases show a change from the expected values for rickets towards tetany. They indicate that at the end of the experiment, when approximate equilibrium had been established, the acidity or alkalinity of the diet has a marked effect. Increasing acidity results in depression of the phosphate of the serum—increasing alkalinity results in depression of the calcium. The condition closest to normal is that caused by the neutral diet. The alkaline diets give a blood picture associated with tetany. The acid diets tend toward the conditions found in the controls—low phosphorus rickets.

2. Histologic Examination of Bones.—Marked healing of the ricketic process occurred in all of the animals except those that died after 1 or 2 days on the altered diet. Slight variations exist between the different groups, also in different slides from the same group. All show marked thinning and resorption of cartilage, orderly trabeculæ, and good calcification—evidence of advanced healing.

3. Analyses of Bones.—The femora were analyzed by the technique of Chick and Roscoe. The results are given in Table II. The greatest percentage of ash is present in the animals which received the neutral diet. The alkaline additions resulted in a bone definitely less good, and the acid diets show the least ash—hardly more than the ash of the controls. Steenbock (19) bases all his conclusions upon the percentage composition of the bones. This method is valuable in that it eliminates the difference due to varying size of the bones. However, it precludes a comparison of the actual weights of the bones and the ash values. These, given in Table II, show further, that the

TABLE I.

Blood Serum Values after Addition of Phosphate to Rickets-Producing Diet.

	Ca	P	Ca:P in food.
	mg. per cent	*mg. per cent*	
Na_3PO_4, 859 cc. 0.1 N alkali*	7.7	8.0	1.0
NaH_2PO_4, 311 cc. 0.1 N alkali*	8.2	7.6	0.95
H_3PO_4, 37 cc. 0.1 N alkali*	11.9	7.1	1.04
$H_3PO_4 + HCl$, 313 cc. 0.1 N acid*	9.0	5.8	1.05
Control, Steenbock diet (17), 35 days	9.8	3.8	4.25

* In these experiments, after 21 days on the rickets-producing diet, rats were given additions of the same amount of phosphorus but in the form of Na_3PO_4, H_3PO_4, and $H_3PO_4 + HCl$ to give the indicated acidity per 100 gm. of diet, for 2 weeks.

neutral diet produces the heaviest bone and that the acid diet produces the lightest bone.

4. Metabolism of Calcium and Phosphorus. Weights of Animals.—At the time when the diet was changed the rats weighed from 63 to 74 gm.—approximately the same as those previously reported. Their gains under the new regimens were quite different. In the ricketic controls there was an average loss of 5 gm.; the rats on the alkaline diets and those on the acid diets gained 0.5 to 1.0 gm., those on the neutral diet gained 16.0 gm.

Food Intakes.—The food intakes did not show so distinct a divergence. On $H_3PO_4 + HCl$ diet the food intakes per rat per day for Periods I and II were, respectively, 5.1 and 5.0 gm.; for the H_3PO_4 diet, 7.5 and 5.6 gm.; for the Na_3PO_4 diet, 5.1 and 5.9

gm.; and for the ricketic control group, 4.7 gm. Thus the gains in weight are not proportional to the food intakes.

Weight of Feces.—The weights of the dried feces calculated per rat per week are more nearly proportional to the food consumption. The feces for the group on H_3PO_4 + HCl weighed respectively for Periods I and II, 4.0 and 3.6 gm.; for the H_3PO_4 group, 5.2 and 3.9 gm.; for the Na_3PO_4 group, 2.6 and 3.7 gm.; and for the control group, 4.0 gm. The feces of the most alkaline group were small compared to the food intakes.

TABLE II.

Analysis of Bones per Single Femur.

Age.	Wet weight.	Dry weight.	Fat-free weight.	Ash.	Ash, fat-free bones.	Diet.
days	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	
65		176	146	84	57.4	Sherman Diet B (16), 35 days. Normal control.
61		107	90	28	31.6	Steenbock diet (17), 35 days. Rickets control.
	280	122	111	38	34.6	Steenbock diet + lettuce, 35 days.
68	294	156	121	57	47.5	" " + Na_3PO_4, 859 cc. 0.1 N alkali.*
					46.2	Steenbock diet + NaH_2PO_4, 311 cc. 0.1 N alkali.*
69	322	175	160	91	57.1	Steenbock diet + H_3PO_4, 37 cc. 0.1 N alkali.*
65	237	114	101	38	37.6	Steenbock diet + H_3PO_4 + HCl, 313 cc. 0.1 N acid.*

* These animals were placed on Steenbock's rickets-producing diet for 21 days; then for the subsequent 14 days they were all given additions of 0.850 gm. of phosphorus in the form of Na_3PO_4, H_3PO_4, and H_3PO_4 + HCl to give the indicated acidity per 100 gm. of diet.

Paths of Excretion.—The intake and output of calcium and phosphorus are given in Table III. Compared with the ricketic controls, the rats show a diminution in urinary calcium and an increase in urinary and fecal phosphorus. All of the periods with added phosphate yield values that are alike but that differ from those obtained during the control period. In that period the

calcium was in excess in both urine and feces; after phosphate is added to the diet the phosphates are in excess in the urine and the calcium is only slightly in excess in the feces. Further, the calcium which in the rickets period had been excreted largely by the bowel is excreted almost entirely by that means, and the phosphorus which had formerly been excreted by the feces alone is now excreted by the urine also. The finer differences in the paths of excretion with regard to the effect of acid and alkali

TABLE III.

Metabolism of Calcium and Phosphorus.

Figures in terms of one rat per week.

Period.	Calcium.						Phosphorus.							
	Intake.	Urine.	Stool.	Spill.	Total output.	Balance.	Intake.	Urine.	Stool.	Spill.	Total output.	Balance.	Ca:P	
wks.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
I	362	17.7	151.2		169	+193	372	46	109	±0	155	+217	0.89	Na_3PO_4
II	361	15.6	265	+7	288	+73	360	46	140	±9	195	+165	0.44	
I	520	9.0	300	±0	309	+211	484	103	173	±0	276	+208	1.02	H_3PO_4
II	400	14.0	275	±0	289	+111	382	65	173	±0	238	+144	0.77	
I	340	11.0	282	±0	292	+48	325	29	159	±0	188	+137	0.35	H_3PO_4
II	337	7.0	273	+2	282	+55	321	108	162	±0	270	+51	1.08	+ HCl.
I	333	58	193	±0	251	82	77	3	53	±0	56	21	3.9	Rickets control.

upon the partition of calcium and phosphorus have been discussed elsewhere (Shohl and Sato (20) and Givens and Mendel (21)). Variations in experiments are such that the quantitative relationships must be evaluated with great caution. Distribution of calcium and phosphorus between the urine and feces is primarily dependent upon the amount and proportion of calcium and phosphorus in the diet.

Balances of Calcium and Phosphorus.—When phosphate is added to the rickets-producing diet the balances of calcium and

phosphorus show marked alterations from those obtained upon the control diet. On the rickets-producing diet, the retention shows marked excess of calcium over phosphorus. After the addition of phosphate, phosphorus retention is in large excess. This is clearly shown by the ratio of Ca:P in the balance. Instead of being 3.0, it is 1.0 or less, indicating a high phosphorus retention. When the balances are computed in terms of actual retention, the calcium balances increase also. As the rats ate slightly differing amounts, comparison between the retentions is more striking when they are calculated as percentage of the intake. The calcium retention which averaged 21 per cent of the intake during the rickets period increases to as much as 50 per cent for the alkaline and neutral diets. The acid diets show only 14 to 16 per cent calcium retention. Similarly for the phosphorus, which in the ricketic control period was 20 to 30 per cent, the retention increases to 38 and 62 per cent for a much larger intake of phosphorus.

Retention of calcium and phosphorus varies with the acidity of the diet. The highest balances are obtained with the neutral diets, slightly less with the alkaline diets, and least for the acid diets. Though the acidity of the diet has a secondary effect, the relative retention of calcium and phosphorus in the body, which is a true measure of the type of bone being laid down, shows that with all the diets, when phosphate is added, a relative excess of phosphorus is retained.

DISCUSSION.

The clinical picture and blood serum studies show marked differences depending upon the degree of acidity. The alkaline diets, according to the blood analyses after 2 weeks, tended toward tetany; the acid diets toward rickets. The important point is, not the values after equilibrium had been attained, but the changes prior to that time. The most severe tetany was produced not by the most alkaline diet but by the neutral diet.

Two conflicting explanations of the effect of the alkalinity of the diet present themselves. The absorption of calcium and phosphorus are dependent upon the acidity. Although the factors governing the acidity of the intestinal tract are important the conditions that govern them are still not well understood

and require further study. We do not know whether calcium and phosphorus may be absorbed as $Ca_3(PO_4)_2$ or as $CaHPO_4$. Steenbock, Hart, Sell, and Jones (22) state: "It is undoubtedly the acidity which makes possible the assimilation of the difficultly soluble calcium salts from the intestine." Babbott, Johnston, and Haskins (23) attribute the appearance of infantile tetany to lessened acidity of the intestine. If alkali causes less rapid absorption of calcium and phosphorus, the maximal effect would be obtained with neutral or acid diet.

In the blood, for a given concentration of phosphate, increase of alkalinity causes a diminished activity or ionization of calcium (24). Were alkalinity the sole factor one should expect consequences opposite to those obtained; namely, the greatest number of deaths and the highest degree of alkalinity from the most alkaline diets. If both absorption and alkalinity together control the degree of tetany each must limit the other. The increase in tetany as neutrality is approached, which is not the maximum for either absorption or acid-base equilibrium of the blood, suggests the possibility of some such mechanism.

The conditions which produce the best final result, cause the worst immediate effects. The cure is so fulminating that death results from too rapid assimilation of phosphorus. To secure optimal conditions for healing, the change to the neutral diet must be gradual. This should be important in establishing therapeutic procedures.

The blood serum values for calcium and phosphorus do not measure the degree of bone changes, as has been emphasized by Steenbock (19) and others. Inasmuch as they reflect only the condition at the moment and are subject to fluctuations apparently due to acid-base conditions, one is forced to rely on other confirming evidence, especially when a quantitative answer is sought. Such information is supplied both by the bone analyses and metabolism studies. These both show that the acid diets have resulted in less bone deposition than the neutral diets.

SUMMARY.

1. When the same amount of phosphate is added to a high calcium, low phosphorus, rickets-producing diet so that the result-

ing mixture is alkaline, neutral, or acid, respectively, cure of rickets results in all cases, according to histological studies.

2. Under the conditions in which rickets is thus cured the composition of the blood serum shows the characteristics of tetany with the alkaline diets and of rickets with the acid diets. The clinical picture shows the characteristics of tetany, occasionally in the alkaline diets, always in the neutral diets, and never in the acid diets.

3. Analyses of the bones show the greatest ash deposition with the neutral diets, smaller with the alkaline diets, and least with the acid diets.

4. The metabolism studies show increase not only in the phosphorus absorption, but also in the calcium absorption. The relative retentions indicate that the body is rapidly recovering from its phosphorus depletion. The largest retentions of calcium and phosphorus are obtained with the neutral diets.

BIBLIOGRAPHY.

1. Zucker, T. F., Johnson, W. C., and Barnett, M., The acid-base ratio of the diet in rickets production, *Proc. Soc. Exp. Biol. and Med.*, 1922, xx, 20.
2. McClendon, J. F., Calcium phosphate metabolism in the diagnosis of rickets, *Am. J. Physiol.*, 1922, lxi, 373.
3. Jones, M. R., Hydrochloric acid therapy in rickets, *J. Am. Med. Assn.*, 1924, lxxxii, 439.
4. Jones, M. R., Studies on inorganic salt metabolism in dogs. III. On certain factors which influence the deposition and resorption of bone, *Am. J. Physiol.*, 1927, lxxix, 694.
5. McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., Studies on experimental rickets. XXII. Conditions which must be fulfilled in preparing animals for testing the anti-rachitic effect of individual foodstuffs, *Bull. Johns Hopkins Hosp.*, 1922, xxxiii, 296.
6. Mellanby, E., Experimental rickets. The effect of cereals and their interaction with other factors of diet and environment in producing rickets, *Med. Research Council, Special Rep. Series, No. 93*, 1925.
7. Shelling, D. H., The production of rickets in rats by changes in the acid-base ratio of the diet. Thesis, Yale University, 1925.
8. Binger, C., Toxicity of phosphates in relation to blood calcium and tetany, *J. Pharmacol. and Exp. Therap.*, 1917, x, 105.
9. Salvesen, H. A., Hastings, A. B., and McIntosh, J. F., Blood changes and clinical symptoms following oral administration of phosphates, *J. Biol. Chem.*, 1924, lx, 311.

10. Gamble, J. L., Ross, G. S., and Tisdall, F. F., Studies of Tetany. I. The effect of calcium chlorid ingestion on the acid-base metabolism of infants, *Am. J. Dis. Child.*, 1923, xxv, 455.
11. Scheer, K., and Solomon, A., Wiekingsweise und Erfolge der Salzsäuremilch bei Tetanie, *Monatschr. Kinderheilk.*, 1923–24, xxvii, 406.
12. Karelitz, S., and Shohl, A. T., Rickets in rats. II. The effect of phosphate added to the diet of ricketic rats, *J. Biol. Chem.*, 1927, lxxiii, 665.
13. Schultzer, P., Studien über Phosphor- und Calciumstoffwechsel bei mangelhaften Kostformen. III. Veränderung des Phosphor- oder Calciumgehalts der Kost, *Biochem. Z.*, 1927, clxxxviii, 435.
14. Karelitz, S., and Shohl, A. T., Rickets in rats. I. Metabolism studies on high calcium-low phosphorus diets, *J. Biol. Chem.*, 1927, lxxiii, 655.
15. Shohl, A. T., and Bennett, H. B., Rickets in rats. III. Metabolism of calcium and phosphorus of rats on restricted food intakes, *J. Biol. Chem.*, 1927, lxxiv, 247.
16. Sherman, H. C., and Campbell, H. L., Growth and reproduction upon simplified food supply. IV. Improvement in nutrition resulting from an increased proportion of milk in the diet, *J. Biol. Chem.*, 1924, lx, 5.
17. Steenbock, H., and Black, A., Fat-soluble vitamins. XXIII. The induction of growth-promoting and calcifying properties in fats and their unsaponifiable constituents by exposure to light, *J. Biol. Chem.*, 1925, lxiv, 263, (274).
18. Sherman, H. C., and Gettler, A. O., The balance of acid-forming and base-forming elements in foods, and its relation to ammonia metabolism, *J. Biol. Chem.*, 1912, xi, 323.
19. Bethke, R. M., Steenbock, H., and Nelson, M. T., Fat-soluble vitamins. XV. Calcium and phosphorus relations to growth and composition of blood and bone with varying vitamin intake, *J. Biol. Chem.*, 1923–24, lviii, 71.
20. Shohl, A. T., and Sato, A., Acid-base metabolism. II. Mineral metabolism, *J. Biol. Chem.*, 1923-24, lviii, 257.
21. Givens, M. H., and Mendel, L. B., Studies in calcium and magnesium metabolism. I. The effects of base and acid, *J. Biol. Chem.*, 1917, xxxi, 421.
22. Steenbock, H., Hart, E. B., Sell, M. T., and Jones, J. H., The availability of calcium salts, *J. Biol. Chem.*, 1923, lvi, 375.
23. Babbott, F. L., Johnston, J. A., and Haskins, C. H., Gastric acidity in infantile tetany, *Am. J. Dis. Child.*, 1923, xxvi, 486.
24. Hastings, A. B., Murray, C. D., and Sendroy, J., Jr., Studies of the solubility of calcium salts. I. The solubility of calcium carbonate in salt solutions and biological fluids, *J. Biol. Chem.*, 1926-27, lxxi, 723.

BLOOD AS A PHYSICOCHEMICAL SYSTEM.

VII. THE COMPOSITION AND RESPIRATORY EXCHANGES OF HUMAN BLOOD DURING RECOVERY FROM PERNICIOUS ANEMIA.

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Since the report by Minot and Murphy (1) of the effect of liver diet on pernicious anemia, it has become readily possible for the first time to study the progression of changes in blood and in the circulatory system during recovery from this condition.

We have made a series of observations on a subject who came into the hospital with a cell count of 1.3 millions per c. mm. and an oxygen capacity of 7.0 volumes per cent. Within 2 months the oxygen capacity of his blood had increased to 16.0 volumes per cent. The case history is given below and in Fig. 1 is shown a record of cell counts and of oxygen capacity determinations during the period he was under our observation.

Three relatively complete sets of observations were made; the first on January 28, 5 days after entry, the second on March 1, the day before discharge from the hospital, and the third on March 23.

These observations were carried out in the usual manner, involving determination of respiratory quotient and rate of blood flow, arterial and venous puncture with experimental derivation of the usual sets of carbon dioxide and oxygen dissociation curves, determination of chloride concentration in equilibrated serum and cells, observations on cell volume, and determination of serum base, serum solids, cell solids, and serum protein. The methods

* National Research Fellow in Chemistry at the time this investigation was made.

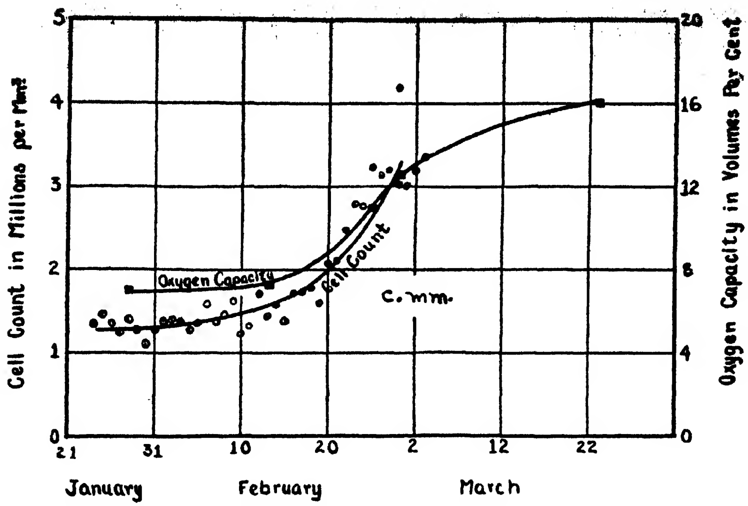

FIG. 1. Oxygen capacity and cell count during recovery from pernicious anemia.

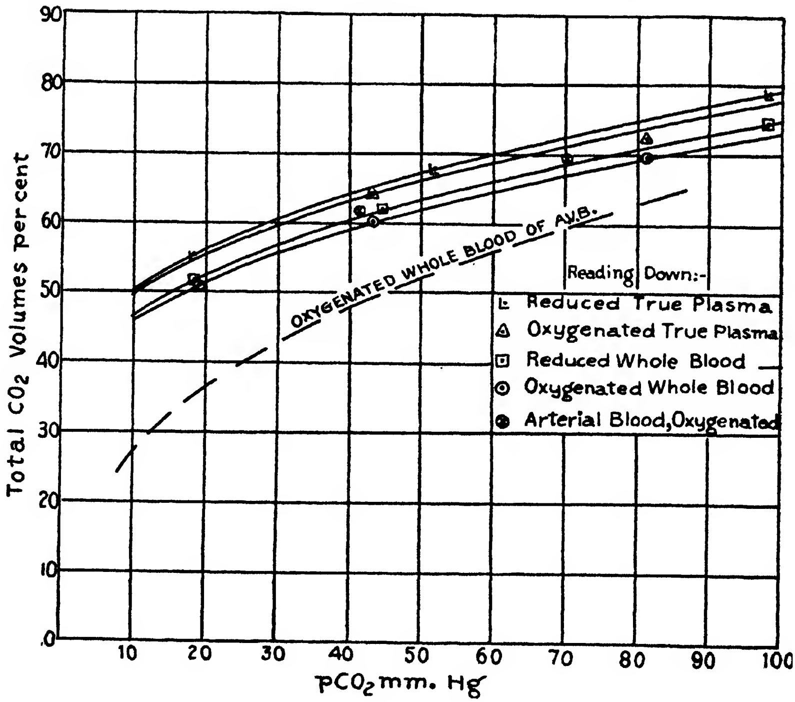

FIG. 2. Carbonic acid dissociation curves on January 28.

employed were the same as those referred to in Paper V (2) and earlier papers of this series.

The carbon dioxide dissociation curves are shown in order in Figs. 2 to 4.

For comparison with blood of normal man, the carbonic acid dissociation curve of oxygenated blood of A. V. B. has been repre-

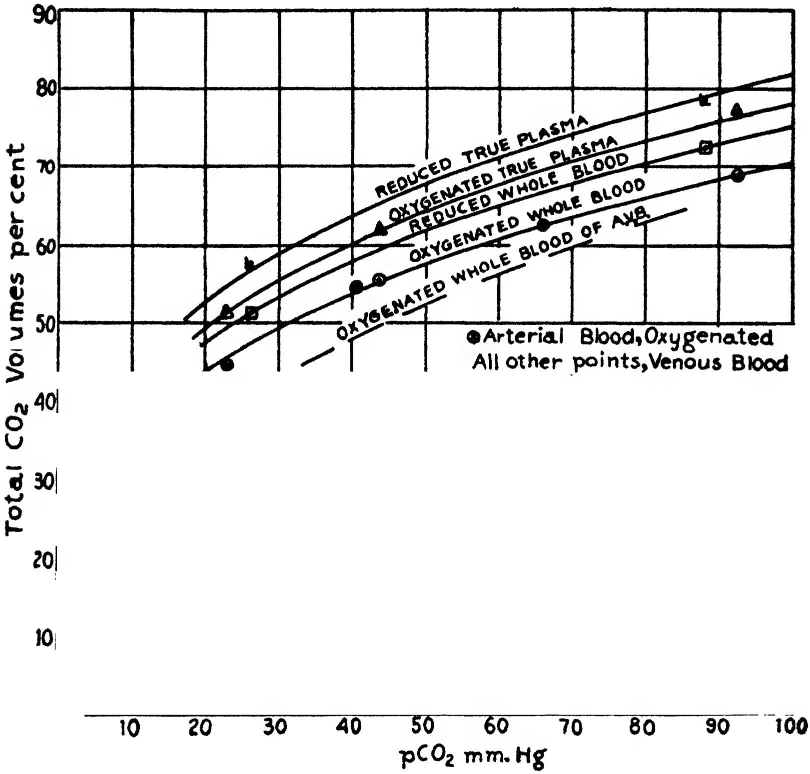

FIG. 3. Carbonic acid dissociation curves on March 1.

sented on each of these figures. It is clear that the slope of these dissociation curves increased as recovery proceeded. The principal factor concerned in this increase of slope is undoubtedly increasing hemoglobin concentration in the blood.

Oxygen dissociation curves based upon each of the same three

specimens of blood are shown in Figs. 5 to 7. Here, too, a comparison may be made with normal man. Oxygen dissociation curves in other cases of pernicious anemia are shown in Fig. 8 together with observations on cell volume, oxygen capacity, and carbonic acid content of oxygenated whole blood at a carbonic acid pressure of 40 mm. of Hg. The position of oxygen dissociation

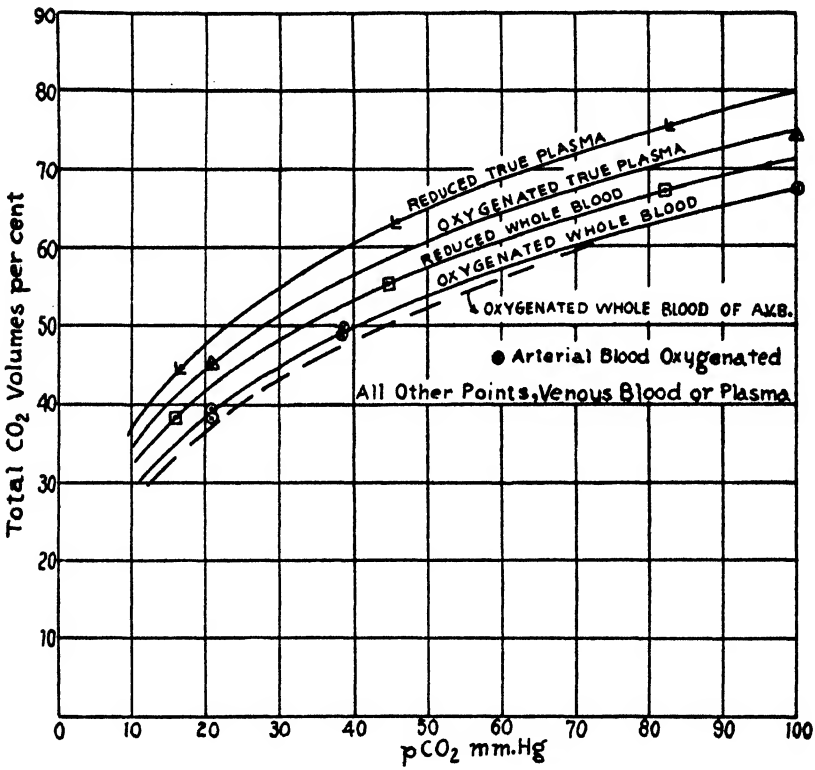

FIG. 4. Carbonic acid dissociation curves on March 23.

curves in pernicious anemia, as defined by the partial pressure of oxygen required for half saturation, is shown in Table I. This table has been compiled from the indicated sources and from Figs. 5 to 8.

It appears from Table I that the oxygen dissociation curves in pernicious anemia are usually appreciably displaced to the right

TABLE I.

Oxygen Dissociation Curves in Health and in Pernicious Anemia.

Subject.	Oxygen partial pressure when HbO_2 = 50 per cent.		
	pCO_2 = 20 mm.	pCO_2 = 40 mm.	pCO_2 = 80 mm.
A. In health.			
	mm. Hg	*mm. Hg*	*mm. Hg*
A. V. B.*	20.0	24.6	32.6
G. S. A.*		26.0	
C. V. C.†	21.0	26.0	33.0
L. M. H.‡		26.5	
D. W. R.§	21.0	26.6	34.8
Average................	20.7	25.9	33.5
B. In pernicious anemia.			
M. C.§		28.0	
H. Sa.§		26.5	
H. St.§	23.5	27.7	
C. G.§		27.7	
A. O.§	23.4	25.0	37.5
H. Sm.§	22.5		
J. T., Mar. 8.	30.0‖	37.0‖	
C. S. M., Apr. 27.		29.8	
O. H. " 27.		29.0	
E. H. " 6.		28.0	
T. J. F., Jan. 28.	21.4	25.0	
Average................	22.5	27.4	37.5
C. In recovery from pernicious anemia.			
T. J. F., Mar. 1.	24.5	29.3	38.0
" " 23.	20.7	26.2	37.0
S. B., Apr. 25.		31.1	

* Bock, A. V., Field, H., Jr., and Adair, G. S., *J. Biol. Chem.*, 1924, lix, 353.

† Dill, D. B., van Caulaert, C., Hurxthal, L. M., Stoddard, J. L., Bock, A. V., and Henderson, L. J., *J. Biol. Chem.*, 1927, lxxiii, 251.

‡ Unpublished data.

§ Richards, D. W., Jr., and Strauss, M. L., *J. Clin. Inv.*, 1927, iv, 105.

‖ Not included in the average.

TABLE II.

Blood Flow and Respiratory Exchanges in Recovery from Pernicious Anemia.

Date.	Weight.	Pulse rate.	Respiratory rate.	Ventilation.	O_2 used per min.	R.Q.	Arterial pCO_2.	Oxygenated venous pCO_2.	O_2 capacity.	Blood flow.	Venous saturation.
A. Subject T. J. F.; height 160 cm.											
1927	*kg.*			*l. per min.*	*cc.*		*mm. Hg*	*mm. Hg*	*vol. per cent*	*l. per min.*	*per cent*
Jan. 28	60	92	15	4.01	232	0.72	41.7	50.5	7.0	6.0	41
Mar. 1	62	65	14	6.15	218	0.92	40.4	48.1	12.5	6.7	69
" 23	61	64	14	5.90	233	0.76	43.1	53.0	16.0	4.3	62
B. Subject J. T.											
Mar. 8		72	19	7.40	254	0.78	36.4*	46.0	5.85	7.3	36

* Alveolar pCO_2.

TABLE III.

Observations on Oxygen Capacity, Cell Volume, and Chloride Distribution. Subject T. J. F.

Date.	O_2 capacity.	pCO_2	pO_2	pH_s	Serum chloride.	Cell chloride.	Cell volume.
1927	*vol. per cent*	*mm.*	*mm.*		*mM per l. serum*	*mM per l. cells*	*per cent*
Jan. 28	7.0	43.3	Air.	7.44	103.0		
		81.6	"	7.21	102.7		14.7
		18.5	5.8	7.75	104.5	37.0	14.5
		52.0	5.2	7.38	102.7		14.6
		98.2	3.7	7.15	101.2		15.3
Feb. 14	7.35						14.3
" 25	11.0						
Mar. 1	12.5	22.9	Air.	7.62	104.8	47.4	29.2
		43.6	"	7.41	103.7		
		92.3	"	7.16	102.5	53.4	30.7
		26.1	8.0	7.61	103.7	48.3	29.0
		87.9	7.6	7.21	102.5	55.6	32.9
Mar. 23	16.0	20.6	Air.	7.61	102.5	49.6	36.7
		100.0	"	7.12	101.5	61.0	38.1
		15.7	5.2	7.72	105.5	49.7	37.1
		44.8	9.5	7.41		59.2	
		82.0	15.2	7.21	100.2	60.2	38.3

and that they do not necessarily return immediately towards normal as recovery proceeds. It appears that the oxygen curves of T. J. F. are exceptional in that they were approximately normal at the time of our first observation. Notwithstanding their

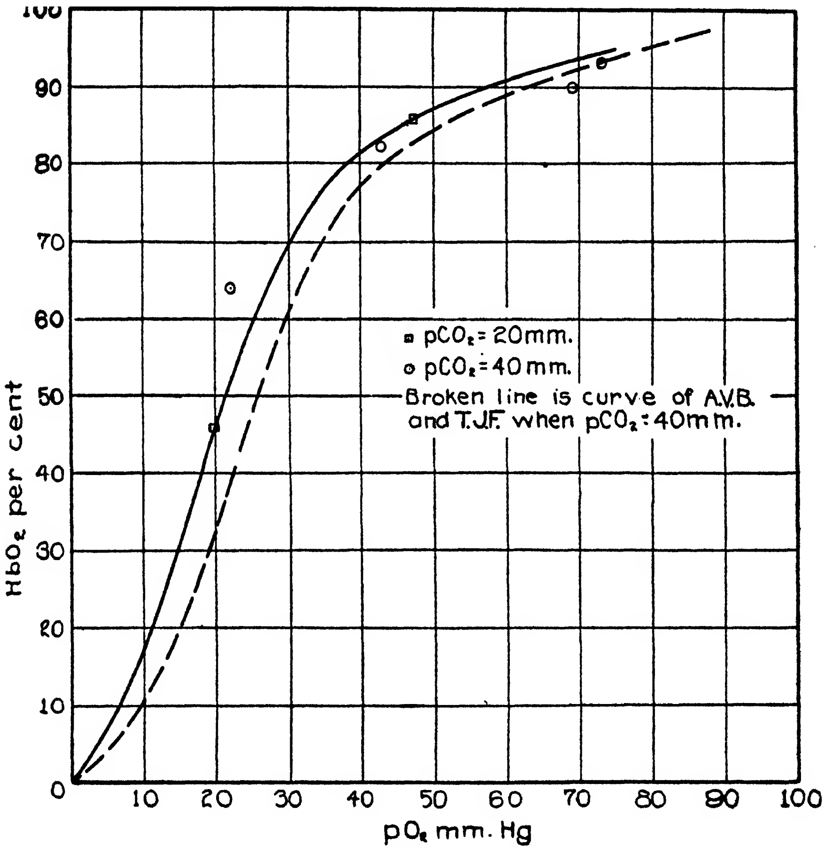

Fig. 5. Oxygen dissociation curves on January 28.

exceptional character, this set of curves has been employed in constructing the first nomogram. That this blood was exceptional in so far as its oxygen dissociation curves were concerned implies that it may have been atypical in other respects. However, nearly all other observations revealed a picture characteristic

of profound anemia and we have no doubt that this first specimen of blood is sufficiently representative for purposes of nomographic description.

Observations on blood flow and respiratory exchanges are assembled in Table II. There is in general a similarity to the

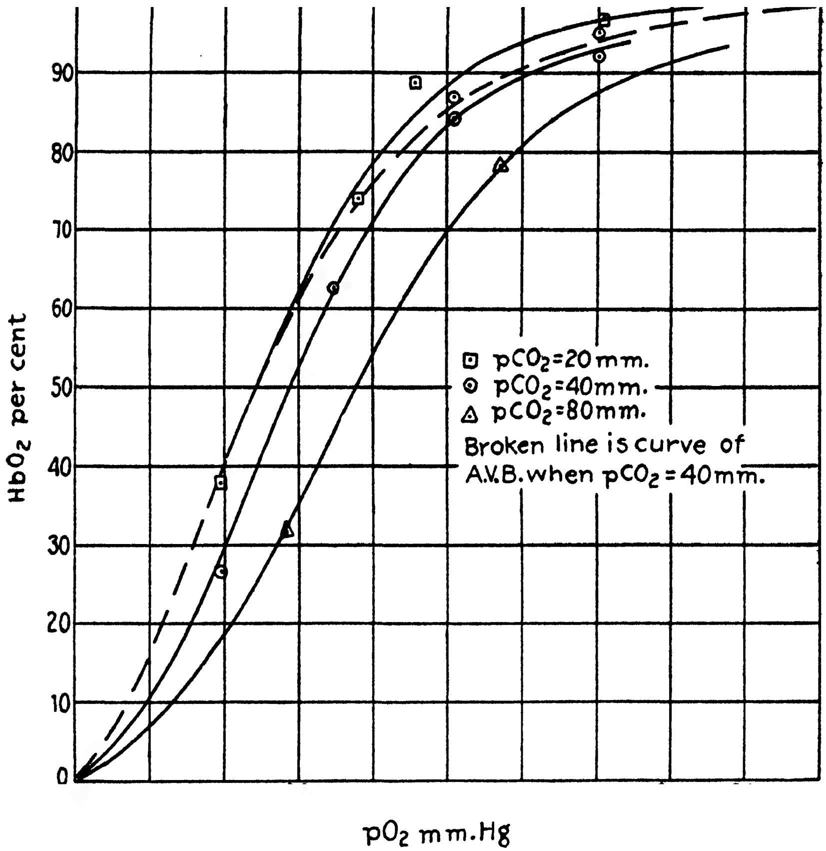

FIG. 6. Oxygen dissociation curves on March 1.

results obtained by Richards and Strauss (3) in their recent study of circulatory adjustment in anemia. The changes observed in our subject as health returned were similar in almost every respect to the changes observed in subject J. K. of Richards and Strauss.

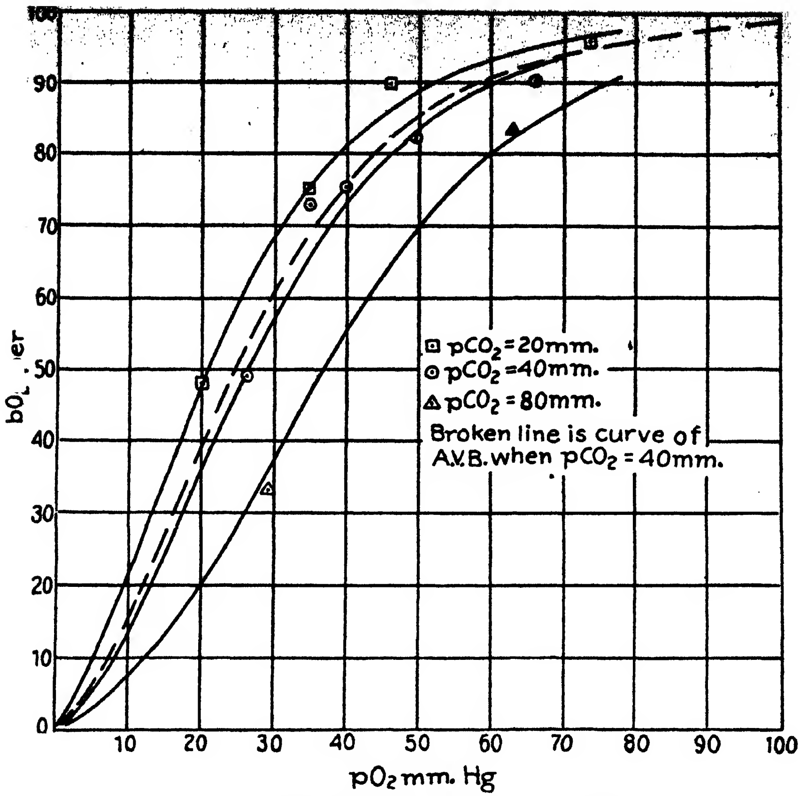

FIG. 7. Oxygen dissociation curves on March 23.

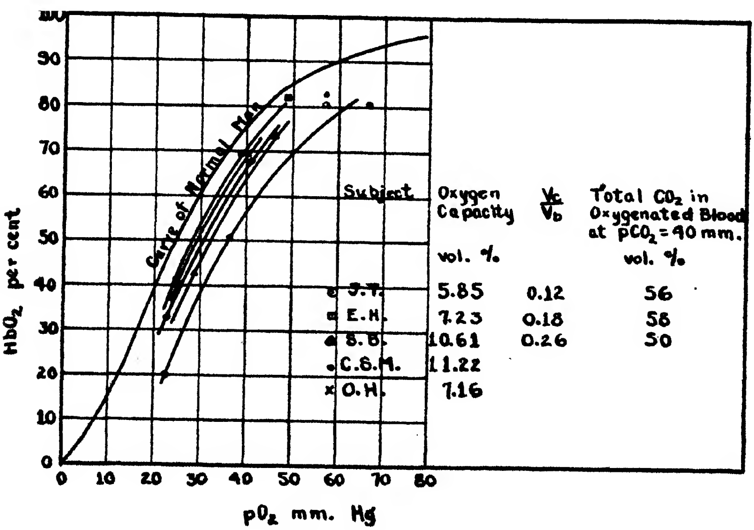

TABLE IV.

Blood of T.J.F. on January 28, 1927.

Concentration of hemoglobin = 3.13 mM per liter of blood.
" " serum proteins = 59.4 gm. " " " "
Respiratory quotient = 0.70

	Arterial.			Venous.			Δ		
	Serum.	Cells.	Whole blood.	Serum.	Cells.	Whole blood.	Serum.	Cells.	Whole blood.
H_2O *cc. per l. blood*	800.6	106.4	907.0	797.4	109.6	907.0	−3.2	+3.2	0.0
B *mM* " " "	124.38	18.75	143.13	124.38	18.75	143.13	0.0	0.0	0.0
X " " " "	1.81	2.97	4.78	2.24	2.86	5.10	+0.43	−0.11	+0.32
Cl " " " "	88.68	5.92	94.60	87.63	6.97	94.60	−1.05	+1.05	0.0
BP " " " "	10.66	7.66	18.32	10.48	6.48	16.96	−0.18	−1.18	−1.36
$BHCO_3$ " " " "	23.23	2.20	25.43	24.03	2.44	26.47	+0.80	+0.24	+1.04
" *vol. per cent*	52.05	4.93	56.98	53.82	5.47	59.29	+1.77	+0.54	+2.31
H_2CO_3, *mM per l. blood*	1.09	0.15	1.24	1.26	0.17	1.43	+0.17	+0.02	+0.19
" *vol. per cent*	2.44	0.33	2.77	2.82	0.39	3.21	+0.38	+0.06	+0.44
Total CO_2, *mM per l. blood*	24.32	2.35	26.67	25.29	2.61	27.90	+0.97	+0.26	+1.23
" " *vol. per cent*	54.49	5.26	59.75	56.64	5.86	62.50	+2.15	+0.60	+2.75
Free O_2, *mM per l. blood*			0.09			0.03			−0.06
" " *vol. per cent*			0.21			0.06			−0.15
Combined O_2, *mM per l. blood*		2.97	2.97		1.28	1.28		−1.69	−1.69
" " *vol. per cent*		6.65	6.65		2.88	2.88		−3.77	−3.77
Total O_2, *mM per l. blood*			3.06			1.31			−1.75
" " *vol. per cent*			6.86			2.94			−3.92
pCO_2, *mm. Hg*			42.0			48.7			+6.7
pO_2 " "			76.0			22.5			−53.5
Volume, *cc. per l. blood*	851.8	148.2	1000.0	848.6	151.4	1000.0	−3.2	+3.2	0.0
pH	7.450	7.100		7.398	7.092		−0.052	−0.008	
r_{theory}			0.713			0.770			+0.057
r_{HCO_3}			0.694			0.738			+0.044
r_{Cl}			0.503			0.579			+0.076

Table II requires but one explanatory notation. Arterial blood was obtained in each experiment on T. J. F. for determination of carbonic acid content and pressure. The values so obtained were used rather than alveolar carbonic acid pressures in calculating rate of blood flow. The other observations and calculations recorded in Table II were made in the usual manner.

TABLE V.

Serum of T. J. F. on January 28, 1927.

		Arterial.	Venous.	Δ
H_2O	*cc. per l. serum*	939.9	939.8	−0.1
B	*mM* " " "	146.00	146.60	+0.60
X	" " " "	2.13	2.64	+0.51
Cl	" " " "	104.08	103.29	−0.53
BP	" " " "	12.51	12.35	−0.16
$BHCO_3$	" " " "	27.28	28.32	+1.04
H_2CO_3	" " " "	1.28	1.49	+0.21
Total CO_2	" " " "	28.56	29.81	+1.25

TABLE VI.

Cells of T. J. F. on January 28, 1927.

		Arterial.	Cells.	Δ
H_2O	*cc. per l. cells*	718.0	723.8	+5.8
B	*mM* " " "	126.54	123.85	−2.69
X	" " " "	20.05	18.89	−1.16
Cl	" " " "	39.94	46.03	+6.09
BP	" " " "	51.70	42.81	−8.89
$BHCO_3$	" " " "	14.85	16.12	+1.27
H_2CO_3	" " " "	1.01	1.12	+0.11
Total CO_2	" " " "	15.86	17.24	+1.38
Combined O_2	" " " "	20.02	8.45	−11.57
Total Hb	" " " "	21.10	20.67	−0.43

Our observations on oxygen capacity, cell volume, and chloride distribution are shown in Table III. We have determined cell volume by centrifugation of equilibrated blood in accurately calibrated 15 cc. Pyrex centrifuge tubes. Equilibrated blood is introduced into such a tube by displacement of paraffin oil. Most of the excess oil is displaced with a 1-hole rubber stopper and

Blood of T.J.F. on March 1, 1927.

Concentration of hemoglobin = 5.58 mM per liter of blood.
" " serum proteins = 47.7 gm. " " " "
Respiratory quotient = 0.918

	Arterial.			Venous.			Δ		
	Serum.	Cells.	Whole blood.	Serum.	Cells.	Whole blood.	Serum.	Cells.	Whole blood.
H_2O *cc. per l. blood*	662.0	215.3	877.3	659.3	218.0	877.3	−2.7	+2.7	0.0
B *mM* " " "	104.82	33.48	138.30	104.82	33.48	138.30	0.0	0.0	0.0
X " " " "	3.71	0.09	3.80	3.51	0.31	3.82	−0.20	+0.22	+0.02
Cl " " " "	74.41	13.43	87.84	73.91	13.93	87.84	−0.50	+0.50	0.0
BP " " " "	8.55	15.03	23.58	8.46	13.90	22.36	−0.09	−1.13	−1.22
$BHCO_3$ " " " "	18.15	4.93	23.08	18.94	5.34	24.28	+0.79	+0.41	+1.20
" *vol. per cent*	40.66	11.05	51.71	42.41	11.97	54.38	+1.75	+0.92	+2.67
H_2CO_3, *mM per l. blood*	0.87	0.28	1.15	0.97	0.32	1.29	+0.10	+0.04	+0.14
" *vol. per cent*	1.94	0.63	2.57	2.18	0.72	2.90	+0.24	+0.09	+0.33
Total CO_2, *mM per l. blood*	19.02	5.21	24.23	19.91	5.66	25.57	+0.89	+0.45	+1.34
" " *vol. per cent*	42.60	11.68	54.28	44.59	12.69	57.28	+1.99	+1.01	+3.00
Free O_2, *mM per l. blood*			0.09			0.05			−0.04
" " *vol. per cent*			0.21			0.11			−0.10
Combined O_2, *mM per l. blood*		5.30	5.30		3.88	3.88		−1.42	−1.42
" " *vol. per cent*		11.87	11.87		8.70	8.70		−3.17	−3.17
Total O_2, *mM per l. blood*			5.39			3.93			−1.46
" " *vol. per cent*			12.08			8.81			−3.27
pCO_2, *mm. Hg*			40.4			45.5			+5.1
pO_2 " "			80.0			41.0			−39.0
Volume, *cc. per l. blood*	703.5	296.5	1000.0	700.8	299.2	1000.0	−2.7	+2.7	0.0
pH	7.438	7.167		7.410	7.157		−0.028	−0.010	
r_{theory}			0.717			0.747			+0.030
r_{HCO_3}			0.836			0.854			+0.018
r_{Cl}			0.555			0.570			+0.015

the hole in the stopper closed with a blunt-ended glass rod. Centrifugation for 60 minutes at about 3000 R.P.M. gives practically constant readings and no significant loss of carbonic acid. The pH_s values shown in Table III are calculated from the Henderson equation, it being assumed $pK' = 6.12$.

TABLE VIII.

Serum of T. J. F. on March 1, 1927.

		Arterial.	Venous.	Δ
H_2O	*cc. per l. serum*	941.0	940.9	−0.1
B	*mM* " " "	149.0	149.58	+0.58
X	" " " "	5.27	5.01	−0.26
Cl	" " " "	105.78	105.48	−0.30
BP	" " " "	12.15	12.07	−0.08
$BHCO_3$	" " " "	25.80	27.02	+1.22
H_2CO_3	" " " "	1.24	1.38	+0.14
Total CO_2	" " " "	27.04	28.40	+1.36

TABLE IX.

Cells of T. J. F. on March 1, 1927.

		Arterial.	Venous.	Δ
H_2O	*cc. per l. cells*	726.0	728.6	+2.6
B	*mM* " " "	112.94	111.89	−1.15
X	" " " "	0.30	1.04	+0.74
Cl	" " " "	45.30	46.58	+1.28
BP	" " " "	50.72	46.43	−4.29
$BHCO_3$	" " " "	16.62	17.84	+1.22
H_2CO_3	" " " "	0.94	1.07	+0.13
Total CO_2	" " " "	17.56	18.91	+1.35
Combined O_2	" " " "	17.87	12.96	−4.91
Total Hb	" " " "	18.82	18.65	+0.17

Observations on serum protein, serum base, serum water, and cell water are included in Tables IV to XII. Cell base and base bound by protein were calculated by means of the equations of Van Slyke, Wu, and McLean (4). Smoothed values for $\frac{V_c}{V_b}$, $\frac{V_s}{V_h}$ and indirectly for $(H_2O)_c$ and $(H_2O)_s$ were obtained by an extension of the equations of Van Slyke, Wu, and McLean reported recently by one of us (5).

TABLE X.

Blood of T.J.F. on March 23, 1927.

Concentration of hemoglobin = 7.14 mM per liter of blood.
" " serum proteins = 42.1 gm. " " " "
Respiratory quotient = 0.76

	Arterial.			Venous.			Δ		
	Serum.	Cells.	Whole blood.	Serum.	Cells.	Whole blood.	Serum.	Cells.	Whole blood.
H_2O *cc. per l. blood*	591.2	273.8	865.0	587.0	278.0	865.0	−4.2	+4.2	0.0
B *mM* " " "	95.90	42.80	138.70	95.90	42.80	138.70	0.0	0.0	0.0
X " " " "	8.23	−2.02	6.21	8.64	−2.15	6.49	+0.41	−0.13	+0.28
Cl " " " "	64.82	20.38	85.20	63.54	21.66	85.20	−1.28	+1.28	0.0
BP " " " "	7.41	18.28	25.69	7.30	16.46	23.76	−0.11	−1.82	−1.93
$BHCO_3$ " " " "	15.44	6.16	21.60	16.42	6.83	23.25	+0.98	+0.67	+1.65
" *vol. per cent*	34.59	13.79	48.38	36.78	15.30	52.08	+2.19	+1.51	+3.70
H_2CO_3, *mM per l. blood*	0.83	0.38	1.21	0.96	0.46	1.42	+0.13	+0.08	+0.21
" *vol. per cent*	1.85	0.85	2.70	2.16	1.02	3.18	+0.31	+0.17	+0.48
Total CO_2, *mM per l. blood*	16.27	6.54	22.81	17.38	7.29	24.67	+1.11	+0.75	+1.86
" " *vol. per cent*	36.44	14.64	51.08	38.94	16.32	55.26	+2.50	+1.68	+4.18
Free O_2, *mM per l. blood*			0.10			0.04			−0.06
" " *vol. per cent*			0.23			0.09			−0.14
Combined O_2, *mM per l. blood*		6.90	6.90		4.49	4.49		−2.41	−2.41
" " *vol. per cent*		15.45	15.45		10.06	10.06		−5.39	−5.39
Total O_2, *mM per l. blood*			7.00			4.53			−2.47
" " *vol. per cent*			15.68			10.15			−5.53
pCO_2, *mm. Hg*			43.1			50.6			+7.1
pO_2 " "			85.0			36.0			−49.0
Volume, *cc. per l. blood*	626.8	373.2	1000.0	622.6	377.4	1000.0	−4.2	+4.2	0.0
pH	7.390	7.127		7.352	7.116		−0.038	−0.011	
r_{theory}			0.733			0.772			+0.039
r_{HCO_3}			0.860			0.878			+0.018
r_{Cl}			0.679			0.720			+0.041

With all this information at hand the nomograms, Figs. 9 to 11, were constructed. From the nomograms and from certain additional experimental observations referred to above, tables were prepared showing the composition of each blood (Tables IV, VII, and X), of corresponding serum (Tables V, VIII, and XI), and of corresponding cells (Tables VI, IX, and XII). The arterial

TABLE XI.

Serum of T. J. F. on March 23, 1927.

		Arterial.	Venous.	Δ
H_2O	*cc. per l. serum*	943.0	942.8	−0.2
B	*mM* " " "	153.0	154.0	+1.0
X	" " " "	13.13	13.88	+0.75
Cl	" " " "	103.42	102.02	−1.40
BP	" " " "	11.82	11.72	−0.10
$BHCO_3$	" " " "	24.63	26.38	+1.75
H_2CO_3	" " " "	1.32	1.54	+0.22
Total CO_2	" " " "	25.95	27.92	+1.97

TABLE XII.

Cells of T. J. F. on March 23, 1927.

		Arterial.	Venous.	Δ
H_2O	*cc. per l. cells*	733.8	736.5	+2.7
B	*mM* " " "	114.70	113.40	−1.30
X	" " " "	−5.41	−5.70	−0.29
Cl	" " " "	54.62	57.39	+2.77
BP	" " " "	48.99	43.61	−5.38
$BHCO_3$	" " " "	16.50	18.10	+1.60
H_2CO_3	" " " "	1.02	1.22	+0.20
Total CO_2	" " " "	17.52	19.32	+1.80
Combined O_2	" " " "	18.49	11.90	−6.59
Total Hb	" " " "	19.13	18.92	−0.21

serums are compared in Table XIII and a similar comparison is made in the case of arterial cells in Table XIV.

With the nomograms and the derived tables at hand ready comparison may be made of certain important variables. Thus the quantity

$$\Sigma = (B) + (X) + (Cl) + (BHCO_3)$$

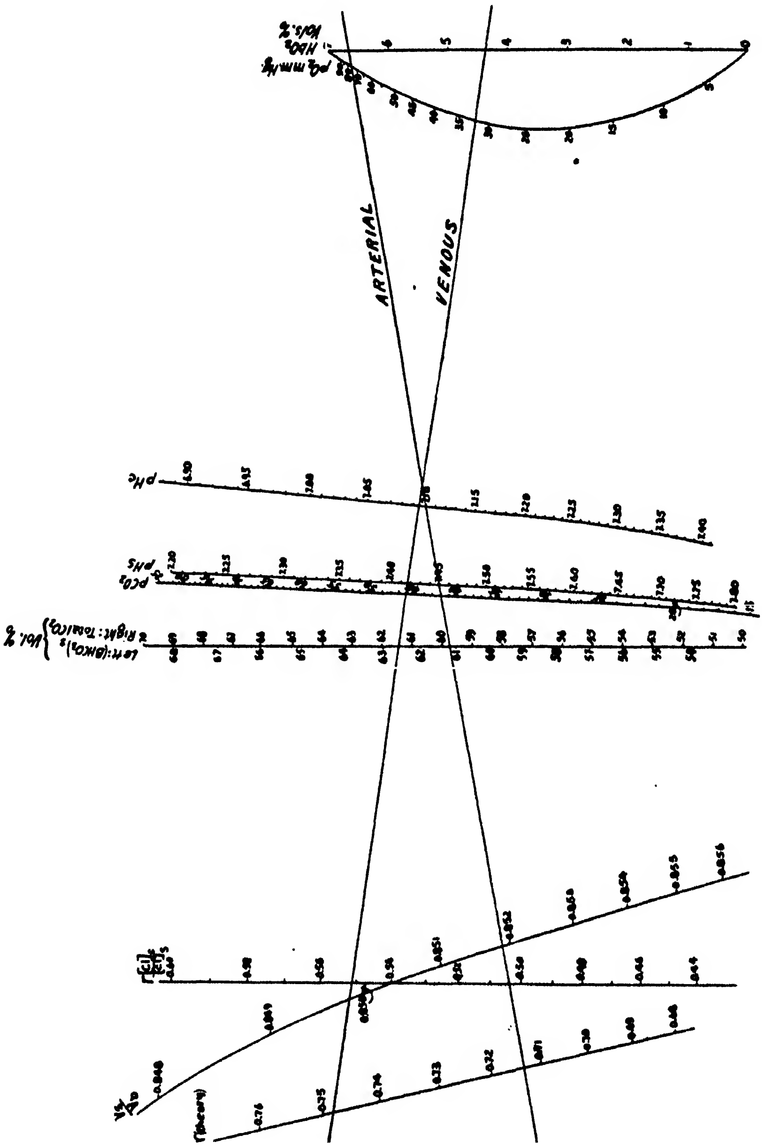

Fig. 9. Blood of T. J. F. on January 28.

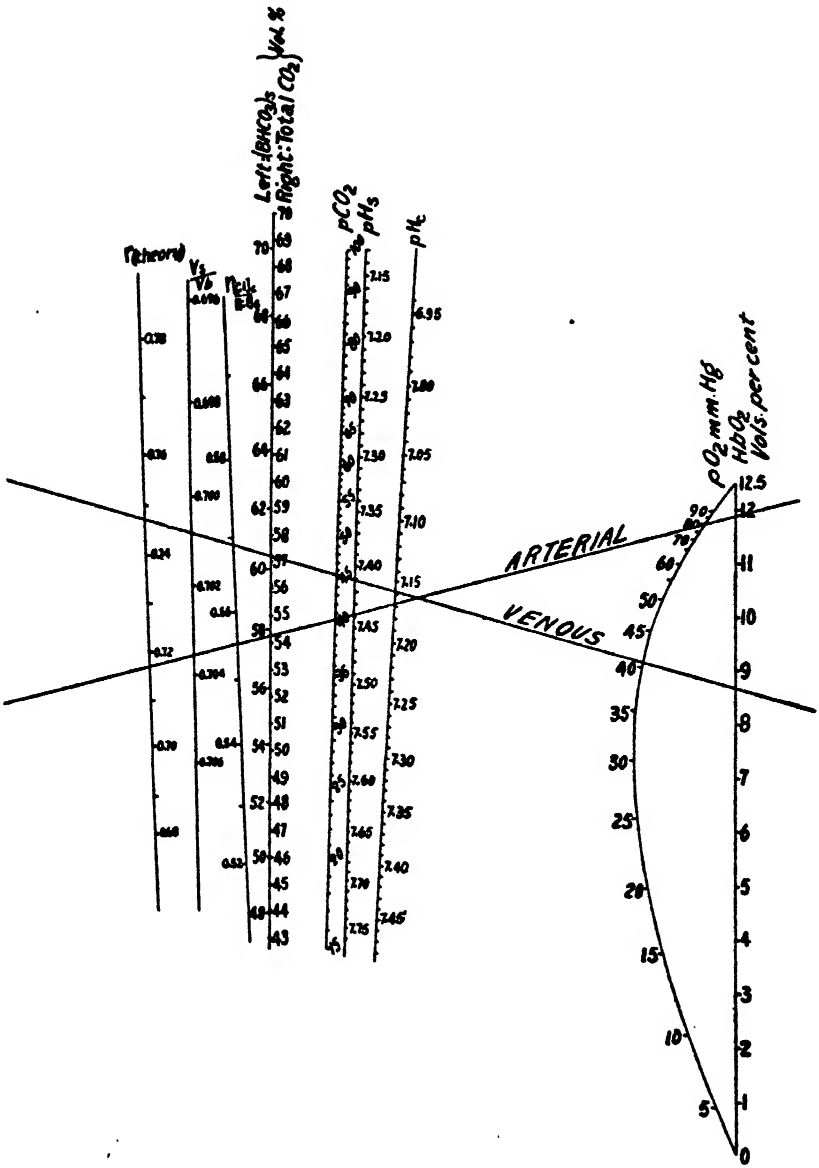

FIG. 10. Blood of T. J. F. on March 1.

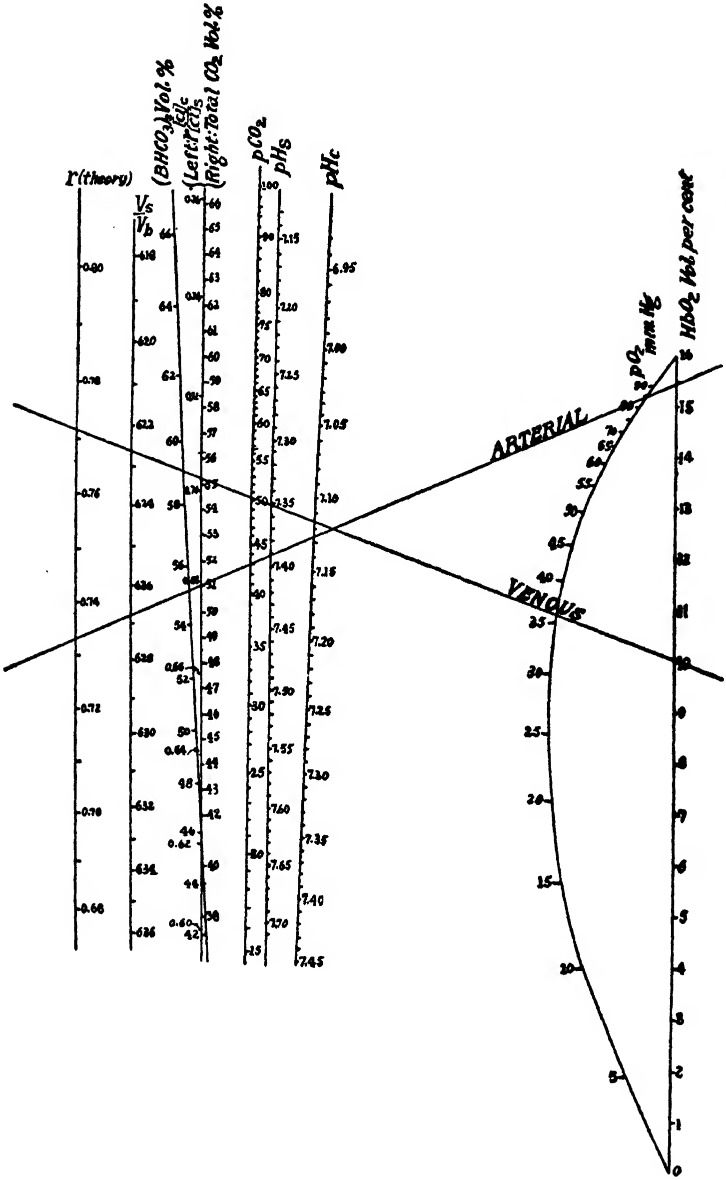

FIG. 11. Blood of T. J. F. on March 23.

which roughly measures the osmotic effect of electrolytes, undergoes the variations indicated in Table XV. On January 28, the electrolyte concentration is low in both serum and cells, the difference between the two phases being normal. Later this concentration returns to normal in the serum but meanwhile in the cells it seems to fall sharply. Even though estimates of this quantity for the cells were of very uncertain accuracy, because indirect, some variation in this quantity is not unexpected and may perhaps be related to the presence of large numbers of reticulated red cells. It is, in fact, hardly open to question that water and electrolytes are

TABLE XIII.

Arterial Serum of T. J. F. during Recovery from Pernicious Anemia.

		Jan. 28. A	Mar. 1. B	Mar. 23. C	A − C	B − C
H_2O	*cc. per l. serum*..........	939.9	941.0	943.0	−3.1	−2.0
B	*mM* " " "	146.00	149.00	153.00	−7.0	−4.0
X	" " " "	2.13	5.27	13.13	−11.00	−7.86
Cl	" " " "	104.08	105.78	103.42	+0.66	+2.36
BP	" " " "	12.51	12.15	11.82	+0.69	+0.33
$BHCO_3$	" " " "	27.28	25.80	24.63	+2.65	+1.17
H_2CO_3	" " " "	1.28	1.24	1.32	−0.04	−0.08
Total CO_2	" " " "	28.56	27.04	25.95	+2.61	+1.09
pH..............................		7.450	7.438	7.390	+0.06	+0.048
Protein, *gm. per l. serum*...........		69.6	67.8	66.9	+2.7	+0.9

associated with nuclear material in proportions quite different from those existing in the cell solution.

It has been observed by Peters, Bulger, Eisenman, and Lee (6) that frequently in severe anemia the concentration of hemoglobin in the cells is significantly decreased. Our observations on this phenomenon are assembled in Table XVI together with similar observations on seven normal subjects, one on a case of diabetic acidosis, and one on the snapping turtle (*Chelydra serpentina*). These observations on normal subjects have been made by J. H. Talbot, H. T. Edwards, together with one of the present authors (D. B. D.), and will be published in more detail elsewhere. It is possible that these two sets of observations are not entirely com-

parable because potassium oxalate was used in the blood of pernicious anemia subjects and heparin was used in all other cases. It appears that potassium oxalate slightly decreases

TABLE XIV.

Arterial Cells of T. J. F. during Recovery from Pernicious Anemia.

		Jan. 28. A	Mar. 1. B	Mar. 23. C	A − C	B − C
H_2O	*cc. per l. cells*	718.0	726.0	733.8	−15.8	−7.8
B	*mM* " " "	126.54	112.94	114.70	+11.84	−1.76
X	" " " "	20.05	0.30	−5.41	+25.46	+5.71
Cl	" " " "	39.94	45.30	54.62	−14.68	−9.32
BP	" " " "	51.70	50.72	48.99	+2.71	+1.73
$BHCO_3$	" " " "	14.85	16.62	16.50	−1.65	+0.12
H_2CO_3	" " " "	1.01	0.94	1.02	−0.01	−0.08
Total CO_2	" " " "	15.86	17.56	17.52	−1.66	+0.04
Combined O_2	" " " "	20.02	17.87	18.49	+1.53	−0.62
Total Hb	" " " "	21.10	18.82	19.13	+1.97	−0.31
pH		7.100	7.167	7.127	−0.027	+0.040

TABLE XV.

Osmotic Effect of Electrolytes in Serum and in Cells.

	T. J. F., Jan. 28.	T. J. F., Mar. 1.	T. J. F., Mar. 23.	A. V. B.
A. Serum.				
Σ	279.5	285.9	294.2	294.9
H_2O	940	941	943	943
$\frac{\Sigma}{H_2O}$	297	304	312	313
B. Cells.				
Σ	201.4	175.2	180.4	211.0
H_2O	718	726	734	705
$\frac{\Sigma}{H_2O}$	280	241	246	299

cell volume and thus increases hemoglobin concentration in the cell. However, there is little doubt that hemoglobin concentration in cells is usually lowered in pernicious anemia. None

of the values observed was as low as some of those found in cases of secondary anemia by Peters and his associates (6), nor found by us in the snapping turtle. Serum and cell water values, although

TABLE XVI.

Cell Volumes and Hemoglobin Concentrations.

Subject.	Remarks.	V_c / V_b	Blood Hb.	Cell Hb.	Cell count.	Serum water.	Cell water.	$\frac{\text{Cell Hb}}{\text{Cell } H_2O}$	Average volume of 1 cell.
			mM per l. blood	*mM per l. cells*	*millions per c.mm.*	*cc. per l. serum*	*cc. per l. cells*	*mM per l.*	*cc. × 10^{-8}*
D. B. D.	Normal.	0.405	7.96	19.7	4.26	941	734	26.8	9.5
J. H. T.	"	0.444	9.06	20.4	4.85	942	723	28.2	9.2
H. T. E.	"	0.475	9.97	21.0	5.20	935	715	29.4	9.1
R. M.	"	0.446	9.20	20.6		937	716	28.8	
A. V. B.	"	0.459	9.26	20.2	4.95	934	734	27.5	9.3
H. H., Jr.	"	0.440	8.95	20.3	4.60	942	716	28.4	9.6
D. W.	"	0.475	9.42	19.8	5.11		726	27.3	9.3
Average.				20.3		938	723		
T. J. F.	Pernicious anemia.	0.148	3.13	21.1	1.30	940	718	29.4	11.4
"	Recovering.	0.297	5.58	18.8	3.15	941	726	25.9	9.4
"	Nearly recovered.	0.373	7.14	19.1		943	734	26.1	
J. T.	Pernicious anemia.	0.123	2.61	21.2					
E. H.	" "	0.179	3.23	18.0	1.65	938	722	24.9	10.9
S. B.	" "	0.257	4.74	18.5	3.00				8.8
Average.				19.3		940	725		
Snapping turtle.		0.200	2.63	13.2		956	723	18.2	
T. B.	Diabetic coma.	0.494	9.82	19.9		925	711	28.0	

somewhat variable, are within the normal range in pernicious anemia, while the volume of the individual cell before recovery began was about 20 per cent greater than normal but fell rapidly as recovery proceeded. This agrees qualitatively with the ob-

servations of Capps (7) on cell diameter and volume index and with observations on diameters by Price-Jones (8) and Bell, Thomas, and Means (9).

Finally there have been assembled in Table XVII values for Van Slyke's r for the bicarbonate, chloride, and hydrogen ions. The values for r_{HCO_3} have been obtained from the nomograms and are thus based on smoothed carbonic acid dissociation curves and

TABLE XVII.

Values for Van Slyke's r in Oxygenated Blood at $pH_s = 7.45$.

Subject.	Remarks.	O_2 capacity.	$(BHCO_3)_b$	$(NaCl)_b$	r_{HCO_3}	r_{Cl}	r_H
		vol. per cent	*mM per l.*	*mM per l.*			
T. J. F.	Pernicious anemia.	7.0	24.8	94.6	0.69	0.50	0.45
"	Recovering.	12.5	22.8	87.8	0.83	0.55	0.66
"	Nearly recovered.	16.0	20.2	85.2	0.84	0.66	0.53
E. H.	Pernicious anemia.	7.2	24.5	91.8	0.79	0.63	0.51
J. T.	" "	5.85	23.9		0.56		0.36
Horse.*	Five experiments.	20.0–23.2			0.75	0.60	0.46
R. M.	Normal man.	20.6	21.9	91.8	0.80	0.66	0.52
Snapping turtle.		5.9	39.0	66.6	0.41	0.42	0.26

* Van Slyke, D. D., Hastings, A. B., Murray, C. D., and Sendroy, J., Jr., *J. Biol. Chem.*, 1925, lxv, 701.

smoothed $\frac{V_c}{V_b}$ values. In the case of r_{Cl} direct determination was made of chloride in cells and in serum by Van Slyke's method, together with experimental determination of $(H_2O)_s$ and $(H_2O)_c$. Values so obtained were plotted as a function of pH_s and the value corresponding to $pH_s = 7.45$ read off. Values for r_H were obtained by calculation from the carbonic acid dissociation curves,

with Henderson's equation with pK′ = 5.93 as found by Van Slyke, Hastings, Murray, and Sendroy (10). On comparing these results with results similarly obtained on the snapping turtle, which has high $(BHCO_3)_b$ values, it turns out that there is a fair correlation between r and $(BHCO_3)_b$. The case of E. F. is quite exceptional so that one must conclude there are other important variables than bicarbonate concentration affecting the heterogeneous equilibrium.

It appears that in pernicious anemia there is usually alkalosis in the serum and relative acidosis in the cells. There is no doubt about the former being true and the evidence for the latter is fairly conclusive. Thus we have the displacement of the oxygen dissociation curves, first observed by Richards and Strauss (3) and confirmed by our observations. Furthermore there is the evidence for a decreased value for r_H both in our experiments and in the observations of Hampson amd Maizels (11). Finally there is the case of J. T. with much the lowest value for r_H and much the greatest displacement of the oxygen dissociation curve. This case approaches that of the snapping turtle in these respects.

SUMMARY.

A study has been made of the changes which occur in the respiratory and circulatory mechanisms of man during recovery from pernicious anemia.

BIBLIOGRAPHY.

1. Minot, G. R., and Murphy, W. P., *J. Am. Med. Assn.*, 1926, lxxxvii, 470.
2. Bock, A. V., Dill, D. B., Hurxthal, L. M., Lawrence, J. S., Coolidge, T. C., Dailey, M. E., and Henderson, L. J., *J. Biol. Chem.*, 1927, lxxiii, 749.
3. Richards, D. W., Jr., and Strauss, M. L., *J. Clin. Inv.*, 1928, v, 161.
4. Van Slyke, D. D., Wu, H., and McLean, F. C., *J. Biol. Chem.*, 1923, lvi, 765.
5. Dill, D. B., *J. Biol. Chem.*, 1928, lxxvi, 543.
6. Peters, J. P., Bulger, H. A., Eisenman, A. J., and Lee, C., *J. Biol. Chem.*, 1926, lxvii, 219.
7. Capps, J. A., *J. Med. Research*, 1903, v, 367.
8. Price-Jones, C., *J. Path. and Bact.*, 1922, xxv, 487.

9. Bell, J. R., Thomas, F. K., and Means, J. H., *J. Clin. Inv.*, 1926, iii, 229.
10. Van Slyke, D. D., Hastings, A. B., Murray, C. D., and Sendroy, J., Jr., *J. Biol. Chem.*, 1925, lxv, 701.
11. Hampson, A. C., and Maizels, M., *J. Physiol.*, 1927, lxiv, p. xx.

Case Histories.

T. J. F., Case 281,274.—An American painter and sign hanger, 37 years old, was admitted January 24, 1927, complaining of shortness of breath and exhaustion. For 3 months he had had dyspnea on exertion, accompanied latterly by a sense of substernal pressure. He had now reached a state of constant exhaustion. His appetite had been fair, meals had been taken at regular hours, and there had been no history of constipation or diarrhea. The diet had included only small amounts of meat. There had been no history of soreness of the tongue. He had had no paresthesia or other nerve involvement. He had had malaria in 1912 and syphilis in 1920 for which he had been adequately treated. He was well developed and nourished, with a dark icteric tint of the skin. The mucous membranes were pale; the tongue clean, pale, with smooth margins. The teeth showed pyorrhea, but no lead line. The rest of the examination revealed nothing abnormal of consequence.

Laboratory Examinations.—Gastric analysis: no free acid, total acidity 5 cc. (0.1 N NaOH). Guaiacum test positive. Blood: red cells 1,300,000, white cells 2500, hemoglobin 45 per cent. The stained preparations showed great variation in the size and shape of the red cells. There were many macrocytes. There was no achromia. The platelets were reduced in number; the reticulated red cells were 1.5 per cent. Urine and stools negative. X-ray study: gastrointestinal tract negative except for some pylorospasm.

On January 28, feeding with a liver extract supplied by Dr. E. J. Cohn was begun, which was subsequently replaced by raw liver. On February 20, the red blood cell count was 2,000,000, white cells 4300, hemoglobin 55 per cent, and reticulated red cells 35 per cent. On March 3, these figures were 3,300,000, 4800, 65 per cent, and 3 per cent. The patient was discharged from the hospital on March 4.

J. T., Case 282,079.—Admitted March 3, 1927. Examined March 8, 1927. On liver diet in the interim.

E. H., Case 282,712.—Admitted April 4, 1927. Examined April 6, 1927.

S. B., Case 282,754.—Admitted April 5, 1927. Examined April 25, 1927. On liver diet in the interim.

C. S. M., Case 283,021.—Admitted April 18, 1927. Examined April 27, 1927. On liver diet in the interim.

O. H., Case 283,092.—Admitted April 21, 1927. Examined April 27, 1927. Blood transfusion April 21. On liver diet April 23 *et seq.*

THE IRON CONTENT OF PLANT AND ANIMAL FOODS.*

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As more data are accumulated it becomes increasingly evident that great variations exist in the mineral content of different samples of the same foodstuff. Only when a large number of samples from different parts of the country have been analyzed will it be possible to approximate the probable mineral content of our diet. With the data now available it is difficult to draw any safe conclusion as to the adequacy or inadequacy of the supply of the mineral elements in an average diet.

In a previous paper (1) the iron content of certain tissues was given. In the present paper figures will be given for the percentage of iron in about 150 of our common food materials. Most of these analyses are of plant materials, but figures for a number of samples of fish and poultry are also included. Included in the averages are the data reported in two previous papers (2, 3) for the iron content of about twenty vegetables.

EXPERIMENTAL.

Preparation of Samples.—The materials used in this study were for the most part bought in the local markets. The fruits and vegetables were carefully washed, spread out in thin layers, and left until the water had evaporated. They were then cut in small pieces and *moisture* was determined by drying to constant weight at 100°. The dried materials were ground in a glass mortar and kept in stoppered bottles until the time of analysis. Before a sample was taken for this purpose the material was dried for several hours to remove any absorbed moisture.

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The cereals were partly broken but not dried before analysis. The nuts were ground but not dried before the sample was taken for analysis. This was necessary in order to avoid a change in the uniformity of the sample due to the separation of fat during the drying. *Moisture* in the cereals and nuts was determined by drying other ground samples to constant weight at 100°.

Methods for the Determination of Iron.—The ferric thiocyanate colorimetric method as described in Standard Methods for the

TABLE I.

Detailed Data for Certain Representative Foods, Illustrating Method for Determination of Iron.

Food. Class.	Food. Sample.	Weight of dry sample.	Fe in standard.	Reading of colorimeter.*	Iron.	Recovery of added iron.	Iron in sample (dry basis).
		gm.	*mg.*	*mm.*	*mg.*	*per cent*	*per cent*
Cereals.	Wheat flour.	8	0.1	27.3	0.073		0.00091
	" " + 0.1 mg. Fe.	8	0.1	11.6	0.172	99	
Fish.	Salmon.	5	0.2	23.2	0.172		0.00344
	" + 0.1 mg. Fe.	5	0.2	14.8	0.270	98	
Fruits.	Peaches.	3	0.1	23.4	0.085		0.00285
	" + 0.1 mg. Fe.	3	0.1	10.8	0.185	100	
Nuts.	Almonds.	4.85	0.3	29.3	0.205		0.00423
	" + 0.1 mg. Fe.	4.85	0.3	19.8	0.303	98	
Vegetables.	Potatoes.	5	0.2	20.7	0.193		0.00386
	" + 0.1 mg. Fe.	5	0.2	13.4	0.298	105	
	Head lettuce.	1	0.1	15.9	0.126		0.01260
	" " + 0.1 mg. Fe.	1	0.1	8.9	0.225	99	

* Standard set at 20 mm.

Examination of Water and Sewage (4) was used when the phosphate content was not so great as to produce fading. To determine whether phosphates or other compounds were interfering, all analyses were run in triplicate and a known quantity of iron (usually 0.1 mg.) was added to one of the three samples. If a recovery of 95 per cent or more was obtained, it was assumed that there was no interference. If a satisfactory recovery was not obtained, the analysis was repeated on a smaller quantity of material. The disturbing effect of phosphates seems to disappear

when the quantity falls below a certain minimum. If the iron content was too low to permit of a reduction in the size of the sample, the analysis was made by the method of Elvehjem and Hart (5).

In Table I are given the complete analytical data for several representative foodstuffs ranging from low to high in their iron content. The recovery of added iron is more than 95 per cent in every case. The same procedure was followed with every sample analyzed and good recovery of added iron was obtained. For this reason we feel that the figures reported in this paper actually express the iron content of these samples.

Iron Content of Foods.—In Table II are given the moisture and iron content of the samples analyzed.

The figures range from 0.00015 per cent of iron for lemon juice to 0.0192 per cent for parsley. The figure for parsley was so suprisingly high that another sample bought a year later was analyzed and was found to contain approximately the same percentage of iron as the first sample. As a carrier of iron, spinach does not compare with parsley. It would be desirable to compare a number of samples of these two vegetables to see whether parsley generally exceeds spinach in its iron content.

If the groups of foodstuffs are arranged in descending order with reference to their iron content, the following series is obtained: Three dried legume seeds, 0.0094 per cent; seven green leafy vegetables, 0.0069 per cent; seven dried fruits, 0.0054 per cent; twelve nuts, 0.0041 per cent; eighteen cereals and their products, 0.0034 per cent; four kinds of poultry, 0.0014 per cent; two green legumes, 0.0014 per cent; fourteen roots, tubers, stalks, and bulbs, 0.0011 per cent; fifteen non-leafy vegetables, 0.00085 per cent; twenty kinds of fish, 0.00083 per cent; twenty-three fresh fruits, 0.00066 per cent.

This order results from calculations made on the basis of the undried edible product. The high rank of legumes, dried fruits, and nuts is, of course, due in large part to the low percentage of moisture contained in these foods. If the calculations are made on the dry basis the leafy vegetables take the first place with more than 6 times as much iron as the next group, the dried legumes. The green leafy vegetables are *par excellence* the best source of iron in the diet.

TABLE II.

Iron Content of Foods (Calculated on Basis of Undried Material).

Food.*	Moisture.	Iron (Fe).
	per cent	*per cent*
Almonds....	3.9	0.00407
Apples.		
Duchess..	86.7	0.00031
Greening.	82.5	0.00061
Yellow transparent.	89.1	0.00022
Snow..............	83.9	0.00058
Apricots, dried (2).	40.7	0.00726
Artichoke..........	84.4	0.00189
Asparagus..........	91.8	0.00079
Bananas...........	75.4	0.00176
Barley...	6.8	0.00358
Beans, Kidney......	12.4	0.00692
" Lima.......	12.3	0.01166
" Navy.......	14.2	0.00952
" String.......		
Maximum........		0.00119
Minimum........		0.00068
Average (7)......	91.4	0.00093
Beets...............	82.3	0.00236
Beet greens, tops..	90.3	0.00355
" " roots.	87.2	0.00183
Blackberries.......	84.1	0.00100
Blueberries........	81.3	0.00041
Bran flakes........	6.5	0.00524
Brazil nuts.........	6.0	0.00393
Brussels sprouts...	87.4	0.00223
Buckwheat........	7.0	0.00320
Butternuts........	3.0	0.00684
Cabbage.		
Maximum.......	93.6	0.00059
Minimum.......	91.0	0.00017
Average (20)....	92.6	0.00034
Cantaloupe (2)....	90.5	0.00051
Carrots...........	90.1	0.00107
Cauliflower.......	91.4	0.00143
Celery............	94.0	0.00077
Celery cabbage.....	94.3	0.00057
Chard........	91.5	0.00402
Cheese, American...	32.3	0.00138
Cherries, black......	81.9	0.00051
red.	88.0	0.00046

* When more than one sample was analyzed, the number of samples is indicated by the figure in parenthesis.

TABLE II—*Continued.*

Food.*	Moisture.	Iron (Fe).
	per cent	*per cent*
Chestnuts, Italian.	34.5	0.00410
Chocolate, bitter...	1.9	0.00315
Cocoa..............	4.5	0.00313
Coconut...........	39.3	0.00267
Corn, white..	5.7	0.00297
yellow.	8.5	0.00228
Corn-meal, yellow.	6.0	0.00130
Corn flakes.........	6.2	0.00278
Cream of Wheat...	7.4	0.00085
Cucumbers.........	96.8	0.00035
Currants (2).......	86.8	0.00070
dried.	32.7	0.00474
Dandelion..........	88.3	0.00604
Dates, dried.......	27.5	0.00507
Eggplant...........	92.5	0.00061
Eggs...............	71.9	0.00252
Egg yolk...........	49.5	0.00760
Figs, dried.........	38.0	0.00396
Fish and sea food.		
Bass.............	77.0	0.00026
Bluefish..........	76.7	0.00060
Catfish...........	80.0	0.00036
Cod..............	81.7	0.00034
Flounder.........	80.0	0.00073
Haddock.........	78.8	0.00042
Halibut..........	67.3	0.00093
Herring..........	77.6	0.00059
Lake trout.......	79.0	0.00078
Lobster..........	81.1	0.00044
Mackerel.........	77.6	0.00075
Oyster (2).......	87.5	0.00314
Perch............	80.4	0.00048
Pickerel..........	72.5	0.00080
Pike.............	80.2	0.00034
Red snapper..	79.2	0.00040
Salmon.......	75.7	0.00083
Shad..........	69.8	0.00053
Shrimp.......	70.4	0.00267
Whitefish.....	79.8	0.00042
Flour, Graham.		0.00370
" patent...	8.9	0.00091
rye.	6.4	0.00283

TABLE II—*Continued.*

Food.*	Moisture.	Iron (Fe).
	per cent	*per cent*
Gooseberries	90.1	0.00047
Grapefruit, pulp	92.8	0.00027
Grapes, Concord.		
Pulp	72.5	0.00074
Skin	77.2	0.00136
Grapes, Malaga	79.6	0.00228
" red	83.2	0.00090
Hazelnuts	3.8	0.00450
Hickory nuts	2.9	0.00238
Hominy	7.5	0.00054
Honey		0.00115
Kohlrabi (2)	90.7	0.00068
Kumquats	85.0	0.00051
Lemon, juice	96.0	0.00015
" peel	87.5	0.00075
Lettuce, head	96.6	0.00042
" leaf	94.4	0.00187
Milk (20)	87.5	0.00024
Molasses	26.2	0.00797
Mushrooms	71.2	0.00314
Oatmeal (2)	6.4	0.00380
Oats	6.2	0.00840
Olives, green, canned	77.0	0.00211
Onions	93.7	0.00030
Orange, juice		0.00028
" peel	79.2	0.00042
" pulp (2)	87.0	0.00066
Oyster plant	76.5	0.00124
Parsley (2)	87.6	0.01921
Parsnips	82.7	0.00107
Peaches	87.2	0.00036
" dried	37.4	0.00606
Peanuts	2.0	0.00231
Pears	83.9	0.00046
Peas, green (3)	75.2	0.00177
Pecans	2.3	0.00258
Peppers, green	94.0	0.00041
" red	91.7	0.00060
Pineapple	92.0	0.00032
Pistachio nuts	4.0	0.00792
Plums (3)	84.9	0.00077
Pomegranate	73.5	0.00117

TABLE II—*Concluded.*

Food.*	Moisture.	Iron (Fe).
	per cent	*per cent*
Potatoes.	78.2	0.00085
sweet.	72.1	0.00092
Poultry.		
Chicken, dark meat.	67.5	0.00101
light	76.6	0.00070
Duck..............	43.7	0.00171
Goose..............	57.0	0.00202
Turkey, dark meat.	72.1	0.00204
" light " .	72.2	0.00103
Prunes, dried.........	44.1	0.00517
Puffed Rice...........	10.9	0.00107
Puffed Wheat.........	8.6	0.00410
Pumpkin.............	91.7	0.00110
Quinces..............	82.5	0.00101
Radishes (2)..........	94.4	0.00136
Raisins, seeded........	28.2	0.00699
" seedless......	31.9	0.00413
Raspberries (2).......	84.1	0.00099
Rhubarb........	94.4	0.00086
Rice, polished.....	9.5	0.00105
Rutabagas........	80.9	0.00107
Rye...............	6.4	0.00370
Spinach...........	81.9	0.00660
Squash, Hubbard.	90.4	0.00055
" Queen....	81.1	0.00039
Strawberries (2)..	90.3	0.00066
Sweet corn (2)....	81.7	0.00051
Tangerines, pulp..	86.0	0.00061
Tomatoes.........	94.2	0.00060
canned..		0.00130
Tomato puree, canned.		0.00220
Turnips................	91.5	0.00070
Walnuts, Black.	2.0	0.00598
English.	3.3	0.00214
Watercress..	92.5	0.00721
Watermelon.	92.7	0.00023
Wheat.......	7.6	0.00372
bran.	3.4	0.00852

Attention is called to the low percentage of iron in cabbage, celery, and head lettuce as compared with the green leafy vegetables. A direct relation between iron content and presence of chlorophyll is evident. From the point of view of its iron content it is unfortunate that head lettuce is chosen by the public generally in preference to the superior leaf lettuce.

That soil and climatic conditions largely determine the iron content of the plant is shown by the great variations which exist in different samples of the same plant material; for example, apples, string beans, and cabbage. One sample may contain 2 or 3 times as much iron as another sample of the same product.

In a previous publication (1) it was noted that beef juice contained only a small percentage of the total iron in the original beef. The same tendency of iron to cling to the solid part of the material is observed in the case of orange juice and pulp. When orange juice was pressed out and strained as is the practice in preparing this material for feeding infants, only about 25 per cent of the total iron was obtained although more than 60 per cent of the weight of the orange pulp was represented by the expressed juice. Tomato juice similarily prepared contained about 38 per cent of the iron in the original vegetable. These facts indicate the desirability of feeding infants both juice and pulp as early as possible in order to take full advantage of the iron content of these materials.

An interesting condition was found to exist in the iron content of various kinds of fish. Salt water fish contained about 40 per cent more iron than the fresh water species. Fish with dark-colored tissue contained about 75 per cent more iron than those having light-colored tissue. The higher iron content of the dark-colored tissues was encountered in both the salt and the fresh water species.

If the figures for the different groups of foods are compared with the figures for the same materials given by Sherman (6), it will be found that the averages are about the same for cereals, nuts, and fish. For the most important sources of iron, the fruits and vegetables, our results are in most instances higher. The average for 62 fruits and vegetables is about 80 per cent higher than the figures given by Sherman. It is to be expected that individual samples would differ greatly but it does not seem reasonable that the average for a large number of samples should exhibit such differences. We are inclined to believe that Sherman's figures

are too low as they are compiled from different sources and are, as Sherman himself says, "of all degrees of probable reliability." The need of more analytical data on the mineral content of our foods is strongly emphasized by these differences.

SUMMARY.

The iron content of about 150 samples of our common food materials has been determined. The figures range from 0.00015 per cent for lemon juice to 0.0192 per cent for parsley. Arranged in descending order with reference to their iron content the classes of foods come as follows: dried legumes, green leafy vegetables, dried fruits, nuts, cereals, poultry, green legumes, roots and tubers, non-leafy vegetables, fish, and fruits.

Different samples of the same food material show great variations in their iron content. For example twenty samples of cabbage varied from 0.00017 per cent of iron to 0.00059 per cent. Vegetables containing but little chlorophyll, cabbage, celery, and head lettuce, were found to be low in iron.

The juice of oranges and tomatoes contains less of the total iron than is proportional to the weight of expressed juice; the iron clings to the solids.

Salt water fish contain more iron than fresh water fish. Fish with dark-colored tissue contain more iron than those with light-colored tissue. The dark meat of poultry is likewise higher in iron than the light meat.

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BIBLIOGRAPHY.

1. Elvehjem, C. A., and Peterson, W. H., *J. Biol. Chem.*, 1927, lxxiv, 433.
2. Peterson, W. H., and Hoppert, C. A., *J. Home Econom.*, 1925, xvii, 265.
3. Peterson, W. H., Elvehjem, C. A., and Jamison, L. A., *Soil Sc.*, 1925, xx, 451.
4. Standard methods for the examination of water and sewage, American Public Health Association, New York, 6th edition, 1925, 46.
5. Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1926, lxvii, 43.
6. Sherman, H. C., Chemistry of food and nutrition, New York, 3rd edition, 1926, 573–597.

THE COLORIMETRIC ESTIMATION OF THE HYDROGEN ION CONCENTRATION OF URINE.*

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Until very recently the colorimetric method of Henderson and Palmer (1) has been quite universally employed in estimating the pH of urine. Since the introduction of the sulfonephthalein series of dyes by Clark and Lubs (2) in 1917, these indicators have gradually replaced the indicators employed by Henderson and Palmer. The method was modified by Palmer, Salvesen, and Jackson (3) by making the urine dilution on a smaller scale and introducing phenol red for the pH range between 6.3 and 7.4 and methyl red for the pH range between 4.7 and 6.3. Fiske (4) employed the indicators methyl red, brom-cresol purple, phenol red, and cresol red. Myers and Booher (5) suggested a simple technique of estimating the pH of urine in which application was made of the bicolorimeter (6) and the phthalein dyes, phenol red, brom-cresol purple, and brom-cresol green. Wedges were calibrated for each of these indicators, the range employed for phenol red being pH 6.6 to 8.4, for brom-cresol purple pH 5.2 to 7.0, and for brom-cresol green pH 4.6 to 5.4. The last named sulfonephthalein dye had recently been introduced by Cohen (7) to replace methyl red. Estimations were made at room temperature on urine diluted 1:10 with distilled water. It was then pointed out that so far as the matching of colors with the bicolorimeter went the error should not exceed ± pH 0.02 to 0.04, but it was realized that the factors of temperature, dilution, salt, and CO_2 content of urine

* The data here presented are taken from a Thesis in Chemistry by Edward Muntwyler submitted to the Graduate College of the State University of Iowa, July, 1927, in partial fulfilment of the requirements for the degree of Master of Science.

might introduce a very appreciable error in the determination. Accordingly a study of these factors was undertaken in which the electrometric method was employed as a check. A preliminary report of these observations was made by Muntwyler, Norris, and Myers (8). While this work was in progress Hastings, Sendroy, and Robson (9) made a report covering many of these points. Their colorimetric estimations were made at 38° on urine diluted 1:5. They have employed bicolor standards consisting of a series of paired tubes containing the acid and alkaline forms of the indicator in proportions corresponding to the pH. In spite of the fact that they carefully controlled the factors which might introduce error, they noted considerable divergence from the electrometric values. By subtracting 0.1 pH from the observed colorimetric value their figures agreed within ± 0.1 pH of the true value. They conclude that at a fivefold dilution the correction involved in the determination may be attributed almost entirely to the effect of dilution.

It is ordinarily much less convenient to make color comparisons at 38° than at room temperature, and, although some of our studies have been carried out at 38°, we have directed our efforts particularly to obtaining a correction factor for colorimetric determinations made at room temperature on diluted urine, comparison being made with values obtained electrometrically at 38° on the undiluted sample. The method of Myers and Booher (5) has been modified in several particulars in an attempt to eliminate as far as possible such errors as the effect of dilution and loss of CO_2. As pointed out by Marshall (10), the loss of CO_2 from urine may introduce a considerable error in the determination of pH, particularly where the latter is high. A saline indicator mixture is employed as a diluent for each indicator and set at a definite pH. Although satisfactory colorimetric readings may be made with the bicolorimeter on a large number of urine samples without dilution, this is not always true. On this account the urine is uniformly diluted 1:5. We have adopted the 1:5 dilution of Hastings, Sendroy, and Robson (9) but believe that the use of a saline solution in place of water increases the accuracy of the determination. Since urine samples may vary widely in their salt content, much consideration has been given to the effect of salt on the indicators. Use has been made of the following indi-

cators: phenol red, brom-thymol blue, brom-cresol purple, and brom-cresol green. Where the highest degree of accuracy is not needed it is feasible to omit the use of brom-thymol blue, since with the bicolorimeter it is possible to match the border line colors satisfactorily in the pH range where phenol red and brom-cresol purple meet.

Method.

Collection of Urine.—The urine is collected under oil in Pyrex Erlenmeyer flasks with the aid of long stemmed funnels.

Diluting Fluids.—As a diluent a saline indicator mixture is prepared as follows: To 780 cc. of 0.9 per cent sodium chloride solution, 100 cc. of the desired diluted indicator are added. This, when diluted, gives the same concentration of dye as in the unknown. Brom-cresol purple, brom-thymol blue, and brom-cresol green in 0.04 per cent strength are prepared from 0.4 per cent stock solutions[1] and phenol red in 0.02 per cent from 0.4 per cent stock solution made up as recommended by Clark (11). These mixtures are then adjusted to a definite pH with the aid of minute quantities of strong solutions of NaOH or HCl. For phenol red the mixture is adjusted to pH 7.4, while the brom-thymol blue mixture is set at pH 7.2, the brom-cresol purple at pH 6.2, and the brom-cresol green at pH 5.0. These solutions are preserved in Pyrex saline bottles as recommended by Myers, Schmitz, and Booher (12).

Preparation of Standard Wedges.—Two wedges are prepared for each indicator, one containing an alkaline and the other an acid solution of the indicator. The alkaline wedges employed for phenol red, brom-cresol purple, and brom-thymol blue are filled with M/15 secondary phosphate containing 2 cc. of the corresponding diluted indicator for 20 cc. of phosphate. The acid wedges for these indicators are filled with M/15 primary phosphate to which a similar amount of indicator has been added. The wedges for the brom-cresol green indicator are made up with Clark's phthalate-NaOH mixtures, the alkaline wedge having a pH of about

[1] These are prepared by grinding up the required amount of dye with the molecular amount of NaOH, the amount of alkali needed for each decigram of dye being: 5.7 cc. of 0.05 N NaOH for phenol red, 3.2 cc. for brom-thymol blue, 3.7 cc. for brom-cresol purple, and 3.2 cc. for brom-cresol green.

6.2 and the acid wedge of about 4.0. As above, 2 cc. of the diluted indicator are used for 20 cc. of the buffer mixture. 10 (or 5) cc.

TABLE I.

Buffer Mixtures for Calibration of Wedges.

pH	M/10 KH phthalate.	M/10 NaOH.	pH	M/15 KH_2PO_4.	M/15 Na_2HPO_4.
	cc.	*cc.*		*cc.*	*cc.*
4.0	5	0.04*	6.2	8.09	1.91
4.6	5	1.22	6.3	7.74	2.26
4.7	5	1.50	6.4	7.30	2.70
4.8	5	1.77	6.5	6.82	3.18
4.9	5	2.08	6.6	6.30	3.70
5.0	5	2.39	6.7	5.66	4.34
5.1	5	2.68	6.8	5.08	4.92
5.2	5	3.00	6.9	4.48	5.52
5.3	5	3.26	7.0	3.89	6.11
5.4	5	3.55	7.1	3.34	6.66
6.2	5	4.70	7.2	2.80	7.20
	M/15 KH_2PO_4.	M/15 Na_2HPO_4.	7.3	2.32	7.68
			7.4	1.92	8.08
5.2	9.82	0.18	7.5	1.59	8.41
5.3	9.74	0.26	7.6	1.30	8.70
5.4	9.64	0.36	7.7	1.06	8.94
5.5	9.58	0.42	7.8	0.85	9.15
5.6	9.48	0.52	7.9	0.69	9.31
5.7	9.33	0.67	8.0	0.56	9.44
5.8	9.16	0.84	8.1	0.43	9.57
5.9	9.00	1.00	8.2	0.32	9.68
6.0	8.71	1.29	8.3	0.25	9.75
6.1	8.40	1.60	8.4	0.20	9.80

* The phthalate-NaOH mixtures are made up to a volume of 10 cc.

The Sørensen buffer phosphate standards from pH 5.2 to 7.8, prepared as in the table, have been found to check electrometrically within the limits of experimental error. As will be seen from Table II the molarity of these standards is important.

The M/15 phosphate solutions are prepared from special reagent salts (Merck's are satisfactory). The secondary sodium phosphate is prepared by dissolving 9.47 gm. of anhydrous Na_2HPO_4 in distilled water and making up to 1 liter, while the primary potassium phosphate is similarly prepared from 9.08 gm. of KH_2PO_4.

portions of the different phosphate and phthalate-NaOH mixtures are prepared as given in Table I and 1.0 (or 0.5) cc. of the corre-

sponding indicator added. Readings are then obtained for each set of wedges with the dominant color nearer the eyepiece. By plotting the scale reading against the pH value a calibration curve for each set of wedges is obtained. Phenol red is calibrated between pH 7.0 and 8.0, brom-thymol blue between 6.4 and 7.4, brom-cresol purple between 5.4 and 6.6, and brom-cresol green between 4.6 and 5.4. Standards are made up differing by 0.1 pH. One should check the wedges against new standards every 2 weeks. When the wedges have been carefully calibrated it is probably sufficient to refill them once in 2 weeks with the same freshly prepared buffer solutions.

Colorimetric Readings and Calculation of Results.—Since the majority of urines have a pH occurring in the brom-cresol purple range, this indicator is tried first. 1.6 cc. of the brom-cresol purple indicator-saline mixture are allowed to flow into the cup of the bicolorimeter under oil. Enough urine is drawn under oil into a 1 cc. syringe so that 0.4 cc. may be transferred into the saline in the cup. The solution is then thoroughly stirred with the needle of the syringe and color comparison made. If the color of the brom-cresol purple indicates an acid urine the brom-cresol green system is employed. On the other hand if the color of the brom-cresol purple indicates an alkaline urine either the brom-thymol blue or the phenol red systems may be employed depending upon the degree of alkalinity. By subtracting 0.2 pH from the observed colorimetric value at 25° the true pH of the undiluted urine at 38° as determined electrometrically is obtained to within $\pm$ 0.1 pH. Urine becomes more acid as the temperature is raised by approximately 0.01 pH per 1° and hence slight differences in room temperature may be corrected to 25°.

EXPERIMENTAL.

Since it was felt that with the colorimetric method as outlined the effect of loss of CO_2 was at a minimum and the effect of dilution was stabilized as far as possible, any abnormal deviations between the colorimetric and electrometric values could be explained by the effect of salt on the indicator. The effect of varying salt concentrations on the indicator was thus considered in some detail.

In every case the electrometric estimations were made on the

undiluted specimen. A modified Clark electrode vessel (Pyrex glass) of 2 cc. capacity with a thermometer opening directly into the vessel was employed. Determinations at 38° were made in an air thermostat. The *e* of the calomel cell was calculated from the voltage obtained from 0.1 N HCl to which was assigned a pH value of 1.085 at 38°. All potential measurements were corrected

TABLE II.

Comparison of Electrometric and Colorimetric pH Values in Solutions of Varying Phosphate Concentration.

Phosphate.	$pH_E 25° - pH_C 25°$.*	Indicator.
M		
0.20	−0.10	Phenol red.
0.10	−0.04	" "
0.067	0.00	" "
0.05	0.02	" "
0.033	0.05	" "
0.02	0.09	" "
0.01	0.13	" "
0.005	0.17	" "
0.003	0.20	" "
0.10	−0.02	Brom-cresol purple.
0.067	0.00	" "
0.05	0.055	" "
0.03	0.075	" "
0.02	0.12	" "
0.01	0.15	" "
0.005	0.19	" "

* These figures are an average of a considerable number of determinations covering the whole useful range of phenol red. In the case of brom-cresol purple the determinations were made close to pH 6.0. The divergence is due in large part to the change in electrometric values, the colorimetric pH remaining relatively constant.

to 760 mm. of dry hydrogen. For the comparative study, urine samples were equilibrated with 40 mm. of CO_2 and then placed under oil. Samples of these were taken for the colorimetric and electrometric estimations. It was found that reproducible voltages could be obtained with one or two refills if a CO_2 tension of 40 mm. was introduced into the electrode vessel with the hydrogen.

TABLE III.

Comparison of Colorimetric and Electrometric pH Values. Sodium Chloride Varied in a Given Phosphate Concentration.

Phosphate.	NaCl	pH_c 25°.*	pH_e 25°.	$pH_c - pH_e$.
		Phenol red.		
M	*M*			
0.085	0.050	7.46	7.41	0.05
0.085	0.100	7.44	7.37	0.07
0.085	0.125	7.55	7.46	0.09
0.085	0.150	7.42	7.34	0.08
0.085	0.200	7.40	7.30	0.10
0.085	0.250	7.48	7.36	0.12
0.085	0.375	7.44	7.32	0.12
0.085	0.500	7.41	7.25	0.15
0.043	0.050	7.43	7.44	−0.01
0.043	0.100	7.42	7.40	0.02
0.043	0.125	7.53	7.49	0.04
0.043	0.150	7.39	7.34	0.05
0.043	0.200	7.37	7.29	0.08
0.043	0.250	7.47	7.38	0.09
0.043	0.375	7.45	7.31	0.14
0.043	0.500	7.41	7.26	0.15
0.020	0.050	7.50	7.51	−0.01
0.020	0.100	7.45	7.45	0.00
0.020	0.125	7.50	7.44	0.06
0.020	0.150	7.43	7.39	0.04
0.020	0.200	7.42	7.34	0.08
0.020	0.250	7.44	7.28	0.16
0.020	0.500	7.39	7.20	0.19
0.008	0.050	7.54	7.50	0.04
0.008	0.125	7.45	7.39	0.06
0.008	0.250	7.39	7.28	0.11
0.008	0.500	7.31	7.13	0.18
0.005	0.050	7.42	7.41	0.01
0.005	0.125	7.38	7.33	0.05
0.005	0.250	7.28	7.18	0.10
0.005	0.500	7.25	7.08	0.17

* In the colorimetric estimation 1 cc. of the dilute indicator solution was added to 10 cc. of the phosphate-sodium chloride solutions.

TABLE III—*Concluded.*

Phosphate.	NaCl	pH_c 25°.*	pH_e 25°.	$pH_c - pH_e$.
Brom-cresol purple.				
M	M			
0.057	0.110	6.17	6.11	0.06
0.057	0.220	6.11	5.99	0.11
0.057	0.330	6.02	5.87	0.16
0.033	0.050	6.06	6.06	0.00
0.033	0.110	6.17	6.11	0.06
0.033	0.125	6.02	5.95	0.07
0.033	0.220	6.11	5.99	0.12
0.033	0.330	6.05	5.87	0.18
0.033	0.350	5.91	5.77	0.14
0.020	0.050	6.10	6.10	0.00
0.020	0.125	6.04	5.99	0.05
0.020	0.250	6.00	5.87	0.13
0.020	0.500	5.92	5.71	0.21
0.01	0.050	6.11	6.13	−0.02
0.01	0.125	6.04	6.00	0.04
0.01	0.250	5.99	5.87	0.12
0.01	0.500	5.93	5.71	0.22
0.008	0.050	6.13	6.14	−0.01
0.008	0.125	6.09	6.00	0.09
0.008	0.250	6.01	5.87	0.14
0.008	0.500	5.92	5.71	0.21

It has ordinarily been assumed that dilution has little effect on a well buffered solution. The directions given for colorimetric determinations of urinary pH call for dilutions from 1:5 to 1:25. In this connection the data of Table II are interesting. At 0.06 M, the same strength as used in the wedges, the colorimetric and electrometric values correspond. However, as the molar strength becomes less than 0.067 the electrometric assumes the greater value, while as the molar strength becomes greater the colorimetric assumes the greater value.

In order to see what magnitude of effect the salt would have on the indicator color, various solutions were prepared having variable sodium chloride and uniform phosphate concentrations.

Colorimetric and electrometric values were obtained on these solutions at 25°. The colorimetric values were obtained by making the color comparisons on solutions to which 1 cc. of indicator had been added to 10 cc. of solution. Table III shows the comparison for phenol red and brom-cresol purple. It will be noted that as the concentration of salt increases, the deviation between the colorimetric and electrometric values increases. Lepper and Martin (13) have already made this same observation for phenol red.

To make a system with which the salt effect of the indicator could be studied and have the results comparable to what might be found in urine, solutions of urea, sodium chloride, phosphate, and sodium bicarbonate were prepared. By keeping the concentration of three of the constituents constant the effect of the fourth could be studied. The solutions were equilibrated with 40 mm. of CO_2 and then placed under oil. Electrometric determinations were made at 38° on the undiluted solution. Colorimetric values were obtained both on the diluted and undiluted sample at 25°. The colorimetric values for the diluted samples were obtained by the method employed for urine; *i.e.*, 0.4 cc. added to 1.6 cc. of saline under oil. The other colorimetric value was obtained by adding 1 cc. of indicator to 10 cc. of solution under oil. Table IV gives the result of this study on brom-cresol purple, brom-thymol blue, and phenol red indicators. As the concentration of salt increases, the deviation between the electrometric and colorimetric values also increases. The deviation for the diluted samples is greater than that of the undiluted at the higher salt concentrations. In the concentrations studied urea does not appear to influence the colorimetric value. Since the sodium chloride concentration may vary markedly in urine, one might expect variations in the differences between the electrometric and colorimetric pH values of similar magnitude as with the prepared solution. If such were the case no absolute correction factor could be applied to the urine.

Since emphasis has been placed on the concentration of sodium chloride in the prepared solutions, it has been determined in urine samples along with the colorimetric and electrometric pH. Table V gives the results of this study. As may be seen, in spite of the fact that the salt concentration varied quite widely, there is no clear cut relation between it and the difference between the colorimetric

TABLE IV.

Comparison of Electrometric and Colorimetric pH Values in Varying Concentrations of Urea, Sodium Chloride, Sodium Bicarbonate, and Phosphate.

PO_4	NaCl	Urea.	$NaHCO_3$	pH_c 25° (undiluted) − pH_e 38° (undiluted).	pH_c 25° (diluted 1:5*) − pH_e 38° (undiluted).
Phenol red.					
M	*M*	*M*	*M*		
0.01	0.025	0.375	0.01	−0.01	−0.01
0.01	0.05	0.375	0.01	0.05	0.07
0.01	0.10	0.375	0.01	0.07	0.11
0.01	0.20	0.375	0.01	0.15	0.20
0.01	0.30	0.375	0.01	0.21	0.27
0.01	0.075	0.375	0.015	0.16	0.15
0.01	0.150	0.375	0.015	0.14	0.17
0.01	0.225	0.375	0.015	0.18	0.22
0.01	0.30	0.375	0.015	0.22	0.27
0.01	0.125	0.375	0.005	0.15	0.17
0.01	0.125	0.375	0.015	0.13	0.13
0.01	0.125	0.375	0.02	0.11	0.13
0.01	0.125	0.375	0.025	0.16	0.15
0.01	0.10	0.625	0.01	0.12	0.13
0.01	0.10	0.500	0.01	0.12	0.14
0.01	0.10	0.625	0.01	0.13	0.16
0.01	0.10	0.250	0.01	0.13	0.16
0.01	0.20	0.500	0.01	0.16	0.20
0.01	0.20	0.125	0.01	0.17	0.22
Brom-thymol blue.					
0.01	0.0625	0.3	0.007	−0.03	−0.04
0.01	0.10	0.3	0.007	0.06	0.06
0.01	0.20	0.3	0.007	0.10	0.11
0.01	0.25	0.3	0.007	0.12	0.18
0.01	0.375	0.3	0.007	0.16	0.26
0.02	0.0625	0.3	0.007	0.06	0.04
0.02	0.10	0.3	0.007	0.09	0.12
0.02	0.20	0.3	0.007	0.14	0.18
0.02	0.25	0.3	0.007	0.16	0.24
0.02	0.375	0.3	0.007	0.19	0.30

*Diluted 1 : 5 with 0.9 per cent NaCl solution.

TABLE IV—*Concluded.*

PO_4	NaCl	Urea.	$NaHCO_3$	pH_c 25° (undiluted) − pH_c 38° (undiluted).	pH_c 25° (diluted 1:5*) − pH_c 38° (undiluted).
		Brom-thymol blue—Continued.			
M	*M*	*M*	*M*		
0.02	0.125	0.00	0.007	0.06	0.09
0.02	0.125	0.30	0.007	0.07	0.08
0.02	0.125	0.50	0.007	0.05	0.08
0.02	0.125	0.60	0.007	0.04	0.07
		Brom-cresol purple.			
0.01	0.05	0.125	0.0025	0.01	−0.04
0.01	0.10	0.125	0.0025	0.05	0.04
0.01	0.20	0.125	0.0025	0.12	0.14
0.01	0.25	0.125	0.0025	0.14	0.17
0.01	0.375	0.125	0.0025	0.19	0.25
0.02	0.05	0.125	0.0025	0.01	0.01
0.02	0.10	0.125	0.0025	0.06	0.07
0.02	0.20	0.125	0.0025	0.11	0.15
0.02	0.25	0.125	0.0025	0.14	0.19
0.02	0.375	0.125	0.0025	0.18	0.26
0.03	0.05	0.125	0.0025	0.03	0.02
0.03	0.10	0.125	0.0025	0.06	0.06
0.03	0.20	0.125	0.0025	0.08	0.13
0.03	0.25	0.125	0.0025	0.12	0.18
0.03	0.375	0.125	0.0025	0.15	0.24
0.02	0.10	0.05	0.0025	0.08	0.08
0.02	0.10	0.10	0.0025	0.08	0.06
0.02	0.10	0.20	0.0025	0.06	0.06
0.02	0.10	0.30	0.0025	0.07	0.06
0.02	0.10	0.375	0.0025	0.08	0.06
0.02	0.20	0.05	0.0025	0.12	0.17
0.02	0.20	0.10	0.0025	0.12	0.16
0.02	0.20	0.20	0.0025	0.11	0.16
0.02	0.20	0.30	0.0025	0.11	0.15
0.02	0.20	0.375	0.0025	0.13	0.18
0.02	0.25	0.05	0.0025	0.15	0.20
0.02	0.25	0.10	0.0025	0.15	0.21
0.02	0.25	0.20	0.0025	0.14	0.23
0.02	0.25	0.30	0.0025	0.16	0.21
0.02	0.25	0.375	0.0025	0.14	0.20

TABLE V.

Comparison of Colorimetric and Electrometric pH Values of Urine Specimens Arranged According to the Molarity of the Sodium Chloride.

Specimen No.	NaCl	pH_c 25° (diluted 1:5*).	pH_e 38° (undiluted).	$pH_c - pH_e$.
		Phenol red.		
	M			
1	0.020	7.02	6.78	0.24
2	0.061	7.15	6.89	0.26
3	0.065	7.09	6.88	0.21
4	0.089	7.22	7.05	0.17
5	0.093	7.23	7.00	0.23
6	0.100	7.10	6.87	0.23
7	0.110	7.38	7.10	0.28
8	0.154	7.34	7.06	0.28
9	0.190	7.26	7.06	0.20
10	0.200	7.55	7.33	0.22
11	0.212	7.66	7.39	0.27
12	0.222	7.28	7.22	0.26
13	0.226	7.11	6.83	0.28
14	0.246	7.40	7.06	0.34
15	0.300	7.50	7.22	0.28
		Brom-thymol blue.		
1	0.034	6.58	6.49	0.09
2	0.065	6.56	6.42	0.14
3	0.089	7.22	7.05	0.17
4	0.090	6.99	6.70	0.19
5	0.100	6.72	6.53	0.19
6	0.100	6.97	6.73	0.24
7	0.110	6.98	6.75	0.23
8	0.154	6.83	6.75	0.08
9	0.157	6.74	6.53	0.21
10	0.190	7.30	7.06	0.24
11	0.230	6.98	6.75	0.23
12	0.230	6.88	6.68	0.20
		Brom-cresol purple.		
1	0.051	6.00	5.80	0.20
2	0.058	6.13	5.96	0.17
3	0.065	6.23	6.10	0.13
4	0.085	6.56	6.33	0.23
5	0.093	5.90	5.73	0.17

TABLE V—*Continued.*

Specimen No.	NaCl	pH_c 25° (diluted 1:5*).	pH_e 38° (undiluted).	$pH_c - pH_e$
Brom-cresol purple—Continued.				
	M			
6	0.099	6.14	5.99	0.15
7	0.102	6.40	6.25	0.15
8	0.110	6.32	6.28	0.04
9	0.116	5.77	5.52	0.25
10	0.120	6.01	5.82	0.19
11	0.120	6.42	6.29	0.13
12	0.130	6.03	5.82	0.21
13	0.140	5.74	5.54	0.20
14	0.144	6.28	6.03	0.25
15	0.147	6.62	6.44	0.18
16	0.150	6.42	6.23	0.19
17	0.160	6.20	5.98	0.22
18	0.164	6.68	6.53	0.15
19	0.168	5.83	5.58	0.25
20	0.180	6.14	5.96	0.18
21	0.191	6.30	6.09	0.21
22	0.195	6.35	6.15	0.20
23	0.200	6.23	5.89	0.34
24	0.201	5.69	5.42	0.27
25	0.216	6.16	5.96	0.20
26	0.230	6.21	5.98	0.23
27	0.230	5.85	5.62	0.23
28	0.236	6.07	5.82	0.25
29	0.230	6.05	5.82	0.23
30	0.246	6.45	6.18	0.27
31	0.250	5.87	5.63	0.24
32	0.274	6.12	5.82	0.30
33	0.277	5.91	5.73	0.18
Brom-cresol green.				
1	0.157	5.12†	4.91	0.21
2	0.168	5.09	4.84	0.25
3	0.177	5.09	4.90	0.19
4	0.185	5.21	4.97	0.24
5	0.194	5.16	4.95	0.21
6	0.198	5.18	4.96	0.22
7	0.210	5.20	4.98	0.22

* Diluted 1 : 5 with 0.9 per cent sodium chloride solution containing the respective indicators.

† Colorimetric values for brom-cresol green above 25° corrected to 25° by adding 0.01 pH per 1°.

TABLE V—*Concluded.*

Summary.

Average correction for phenol red −0.24 pH. 12 out of 15 are within ±0.05 pH of the average.

Average correction for brom-thymol blue −0.20 pH. 9 out of 12 are within ±0.05 pH of this value.

Average correction for brom-cresol purple −0.205 pH. 26 out of 33 are within ±0.05 pH of the average. Average correction for Specimens 1 to 17, 0.17 pH; Specimens 18 to 33, 0.23 pH.

Average correction for brom-cresol green −0.22 pH. Maximum deviation in 7 determinations ±0.03 pH of the average correction.

TABLE VI.

Comparison of Colorimetric and Electrometric pH Values of Urine.

Specimen No.	NaCl	pH_c 38°.	pH_e 38°.	$pH_c - pH_e$.	Indicator.
	M				
1	0.048	5.99	5.95	0.04	Brom-cresol purple.
2	0.072	6.15	6.08	0.07	" "
3	0.085	6.41	6.28	0.13	" "
4	0.089	6.13	6.06	0.07	" "
5	0.109	6.12	6.01	0.11	" "
6	0.120	6.27	6.13	0.14	" "
7	0.160	6.35	6.23	0.12	" "
8	0.190	6.37	6.16	0.21	" "
9	0.208	5.95	5.79	0.16	" "
10	0.284	6.10	5.92	0.18	" "
11	0.058	7.14	7.06	0.08	Phenol red.
12	0.085	7.30	7.24	0.06	" "
Average difference.................				0.11	

and electrometric values. However, in the brom-cresol purple series it will be noted that the first seventeen urine specimens with the lowest sodium chloride concentration showed an average difference of 0.17 pH, while the last sixteen specimens with higher salt concentrations showed an average difference of 0.23 pH. The average correction for the brom-cresol purple and brom-thymol blue is 0.20 pH, while that for the phenol red and brom-cresol green is slightly higher; namely, 0.24 and 0.22 pH respectively.

A few determinations, shown in Table VI, were made at 38°

both electrometrically and colorimetrically. The average difference between the two values is 0.11 pH. Using the bicolorimeter at 38° was found to be less convenient than at room temperature, since the instrument must be placed in the thermostat with the eyepiece protruding. It is hence recommended that the determinations be made at room temperature (as close to 25° as possible), slight changes in temperature from 25° being corrected with the aid of Table VII.

TABLE VII.

Changes in Urine pH with Temperature.

Specimen No.	pH_e 38°.	pH_e 25°.	pH_e 25° − pH_e 38°.
1	6.18	6.27	0.09
2	5.63	5.73	0.10
3	6.09	6.15	0.06
4	6.10	6.17	0.07
5	5.73	5.81	0.08
6	5.62	5.70	0.08
Average difference..................................			0.08

DISCUSSION.

While it is true that an error of 0.1 or 0.2 pH in the estimation of the pH of urine is of relatively little significance owing to the wide variations which may be encountered normally (4.8 to 8.0 pH), still in carrying out any determination the error of the method should be known. When this study was undertaken we did not feel that this was true of the estimation of the urinary pH by the colorimetric method. Furthermore, we felt that a study of the colorimetric method as applied to urine might throw some light on the method as used for blood, and other body fluids, also that some of the observations might be applied to bacteriological culture media.

Attention should be called to the fact that the colorimetric value of various solutions may at times deviate quite appreciably from the electrometric value, depending upon the salt concentration (Tables II, III, and IV). For example, when colorimetric and electrometric values are obtained on various strength phosphate solutions, the deviation becomes increasingly great as the

molar strength of the phosphate varies from 0.067 M. When sodium chloride is added to the phosphate solution and the molarity of the latter kept constant, while the concentration of sodium chloride is gradually increased, the divergence between the electrometric and colorimetric methods increases. The addition of urea appears to be without effect. The influence of the molarity of sodium chloride in the urine series (Table V) is not so readily apparent, but nevertheless if the first seventeen determinations in the brom-cresol purple group are compared with the last sixteen determinations, it will be observed that the divergence between the two determinations is 0.17 pH for the urines with the lowest content of sodium chloride and 0.23 pH for those with the highest content. From these observations the use of a saline diluent in place of water would seem obvious.

In studying the colorimetric change of pH on dilution of both urine and prepared solutions it was found that the value obtained depended in some degree upon the pH of the solution in respect to the pH of the diluent. A urine with a high pH diluted with distilled water having a pH of about 6.0 has a different pH dilution curve than that of a urine with a low pH. A 1:5 dilution gave the least deviation and yet seemed to reduce color interference to a minimum. To eliminate as far as possible the variations in pH due to the variation in the pH of the diluent and to stabilize the effect of change in salt concentration on dilution, various saline indicator solutions set at a definite pH have been used. This principle was used by Cullen (14) in his colorimetric method for the estimation of the pH of blood plasma. Dilutions of 10 to 25 times with water as has been done in the past may lead in some instances to considerable error. Hastings, Sendroy, and Robson (9) employ a 1:5 dilution with water and make the color comparison at 38°. This gave them an average correction of 0.1 pH. In the limited number of samples determined at 38° in this work with the saline diluent an average correction of the same magnitude was obtained, namely 0.11 pH, thus confirming their average figure.

While it may seem troublesome to prepare saline solutions for each indicator it saves time where many determinations are to be run. With the use of the special bottles the saline solutions, when once adjusted, will keep for considerable periods. After the saline solutions have been prepared, and the wedges calibrated, check

determinations may be made within 5 minutes, and a hundred or more determinations carried out in the course of a day. Since the determinations are performed under oil and the addition of urine is rapidly accomplished, the loss of CO_2 is minimal. The determinations are carried out at room temperature (25°). In addition to the greater convenience of this, there would seem to be less opportunity for loss of CO_2 than at 38°. When the determinations are carried out as described at room temperature on urine diluted 1:5 with the saline-indicator solution the results, after the correction factor is deducted, agreed in 80 per cent of the cases within 0.05 pH of the correct value of the undiluted specimen at body temperature.

In the matching of colors a very high degree of accuracy may be secured with the bicolorimeter, quite equal to that obtained with the best models of the Duboscq colorimeter. Although modifications of the Duboscq have been suggested permitting the matching of two colors, it is believed that for this work the bicolorimeter has inherent superiorities: The standards are kept in tightly stoppered wedges and are not exposed to the air, and one views the unknown in a horizontal instead of a vertical position thus permitting the unknown to be covered with oil without interfering with the action of the plunger. Since only the more alkaline wedge is calibrated the yellow of the acid wedges may be used, in part, to match out the yellow of the urine still present at the 1:5 dilution.

SUMMARY.

A colorimetric method for estimating the pH of urine is described in which use is made of the bicolorimeter and the phthalein dyes, phenol red, brom-thymol blue, brom-cresol purple, and brom-cresol green.

When a 1:5 dilution with a saline diluent set at a definite pH rather than with distilled water, is employed and the determination made under oil, the effect of dilution is stabilized as far as possible and the loss of CO_2 is minimal.

The determinations are performed at room temperature (25°) and, by means of corrections obtained by comparison with the electrometric method, the values are corrected to 38°. The cor-

rection factor for phenol red is 0.24, for brom-thymol blue 0.20, for brom-cresol purple 0.20, and for brom-cresol green 0.22 pH. In a study of 67 urine specimens with the pH ranging from 4.9 to 7.2 the above corrections brought the colorimetric estimations within 0.05 pH of the correct value in 80 per cent of the determinations.

Consideration has been given to the effect of salt on the indicator and its possible interference in the determination of the pH in urine discussed. As the salt concentration increases above M/15 the colorimetric value is greater than the electrometric, while with concentrations less than M/15 the reverse is true.

BIBLIOGRAPHY.

1. Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1912–13, xiii, 393.
2. Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1, 109, 191.
3. Palmer, W. W., Salvesen, H., and Jackson, H., Jr., *J. Biol. Chem.*, 1920–21, xlv, 101.
4. Fiske, C. H., *J. Biol. Chem.*, 1921, xlix, 163.
5. Myers, V. C., and Booher, L. E., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 511.
6. Myers, V. C., *J. Biol. Chem.*, 1922, liv, 675.
7. Cohen, B., *Proc. Soc. Exp. Biol. and Med.*, 1922–23, xx, 124.
8. Muntwyler, E., Norris, E. R., and Myers, V. C., *Proc. Soc. Exp. Biol. and Med.*, 1925–26, xxiii, 826.
9. Hastings, A. B., Sendroy, J., Jr., and Robson, W., *J. Biol. Chem.*, 1925, lxv, 381.
10. Marshall, E. K., Jr., *J. Biol. Chem.*, 1922, li, 3.
11. Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd edition, 1923.
12. Myers, V. C., Schmitz, H. W., and Booher, L. E., *J. Biol. Chem.*, 1923, lvii, 209.
13. Lepper, E. H., and Martin, C. J., *Biochem. J.*, 1926, xx, 45.
14. Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 501.

THE COLORIMETRIC ESTIMATION OF THE HYDROGEN ION CONCENTRATION OF BLOOD.*

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With the introduction of Cullen's colorimetric method (1) of estimating the hydrogen ion concentration of blood serum or plasma, it seemed possible for every laboratory to have a method employing minimum material and apparatus and yet secure reliable results. It is generally conceded that the results obtained by the method on normal human plasma or serum agree very well with the true pH as determined electrometrically. Recently, however, considerable doubt has arisen as to its applicability to abnormal samples. Austin, Stadie, and Robinson (2) observed considerable variation, under pathological conditions in man and in the dog under normal and experimental conditions, in the difference between the colorimetric reading of the diluted serum or plasma at 20° or at 38° and the true pH of the undiluted sample at 38°. They conclude that for use with pathological sera, or under changing experimental conditions, the C or the H correction must be determined on each serum if the colorimetric readings made at 20° or 38° are to be relied upon, and have outlined a procedure for doing this. Hastings, Neill, Morgan, and Binger (3), studying the blood reaction in cases of pneumonia, find it better to use 0.27 as the correction factor instead of 0.22. Cullen, Keeler, and

* The data here presented are taken from a dissertation by Edward Muntwyler to be submitted to the Graduate College of the State University of Iowa, June, 1928, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

A preliminary report of these observations was presented before the Iowa Branch of the Society for Experimental Biology and Medicine, April 28, 1927 (*Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 789).

Robinson (4) observed for the abnormal sera in their study that the C correction apparently does not have the same degree of constancy that it has for normal human plasma. The application of the method to dog blood appears to be quite questionable. The most striking report in this connection was made by Miss Bennett (5) who concluded that the Cullen colorimetric method is unreliable even for comparative studies after hemorrhage in

Hastings and Sendroy (6) found that the correction used by Cullen for the colorimetric determinations performed at room temperature is unnecessary when the readings are made with the diluted plasma at body temperature. As mentioned above, Austin, Stadie, and Robinson (2) found that this was not consistently the case and report considerable variation in the H correction.

Myers, Schmitz, and Booher (7) have introduced a microcolorimetric method of estimating the hydrogen ion concentration of blood plasma. This method is essentially an adaptation of the Cullen (1) technique to the Myers bicolorimeter (8). With the use of the bicolorimeter they conclude that color comparisons may be made within ±0.02 pH. At the time of their presentation no comparison was made with values obtained electrometrically. In connection with another study (9) we have had the opportunity of comparing the colorimetric values obtained by this method both at room temperature and at 38° with the true pH as determined electrometrically at 38°.

EXPERIMENTAL.

The larger number of the cases studied were peptic ulcer cases receiving alkali. The diagnoses were divided as follows: Duodenal ulcer 52, gastric ulcer 5, pregnancy 10, various forms of malignancy, 10 of which were under x-ray therapy, 23, and miscellaneous 24. Of these 65 were males and 49 females. The blood was drawn under oil without stasis from an arm vein into a 20 cc. syringe and transferred under oil into bulb tubes of 15 cc. capacity containing 3 drops of 20 per cent potassium oxalate (see Fig. 1). With the slight pressure exerted the blood readily takes up the oxalate and does not clot. The tubes were immediately stoppered and centrifuged. The plasma was then carefully separated

under oil by means of a syringe and transferred under oil to a small tube. The tube was tightly stoppered and placed in the refrigerator until the analyses were begun. The samples were usually collected between 6 and 7 a.m. and the plasma separated as soon as possible. With the interference of other work it was sometimes necessary for the samples to remain in the refrigerator for several hours. To observe the change in pH accompanying such a procedure a number of samples were allowed to remain in the refrigerator for considerable periods. As may be seen in Table I the

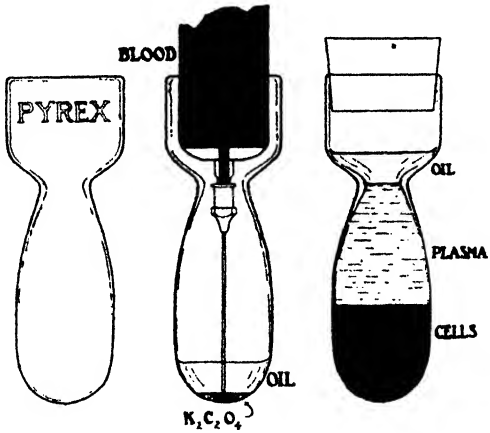

FIG. 1. Showing the plasma separation tubes with bulbs of 15 cc. capacity, which have been employed in these experiments. They allow the use of 20 and 30 cc. glass syringes, thus permitting the withdrawal of sufficient blood for other analyses. Tubes with bulbs of 5 and 10 cc. capacity have also been used.

changes are not very great and hence it was assumed that for the periods of standing in this work the change was negligible. This change would not, of course, enter into this comparative study since the determinations were performed as near simultaneously as possible.

The saline solution employed as the diluent was prepared in the following manner: To 995 cc. of 0.9 per cent sodium chloride solution 105 cc. of 0.02 per cent phenol red solution were added. This mixture was then adjusted to a colorimetric reading of about pH 7.55 with a 1 per cent sodium hydroxide solution, and pre-

served in saline bottles as previously described (7). This saline takes into account the further 5 per cent dilution of the dye introduced by the addition of the plasma and not allowed for by Myers, Schmitz, and Booher (7). 2 cc. of this saline were allowed to flow into the cup of the bicolorimeter under oil. A small portion of the plasma was then drawn up into a 0.5 cc. tuberculin syringe, graduated to 0.01 cc., under oil and 0.1 cc. immediately discharged into the saline in the cup. This solution was stirred with the needle and the color comparison made. The calibration of the wedges

TABLE I.

Changes in Colorimetric pH of Plasma Kept under Oil.

Case.	Initial pH at 20° (corrected).	Length of time under oil.	pH value after standing.	CO_2 content, vols. per cent.	Remarks.
		hrs.			
R. P.	7.78	20	7.79	74.9	Placed in ice box.
"	7.65	16	7.66	50.4	" " " "
L. H.	7.67	18	7.68	65.5	" " " "
"	7.68	17	7.72	77.7	" " " "
M. W.	7.64	17	7.66	66.4	" " " "
O. J.	7.73	17	7.73	74.0	" " " "
M. W.	7.69	15	7.71	60.7	" " " "
"	7.67	3½	7.67	60.7	" " " "
R. P.	7.72	19	7.73	57.9	" " " "
G. F.	7.61	6	7.61	63.6	" " " "
		11	7.65	63.6	Heated at 38° for ½ hr.

and other manipulations were essentially those described previously (7), and similar to those given in the preceding paper (10).

The colorimetric estimations were performed both at room temperature and 38°. The room temperature was obtained by placing a small thermometer in the saline in the cup of the bicolorimeter. The observed colorimetric readings were then corrected to 20° by adding 0.01 pH for every degree above 20°. The colorimetric estimations at 38° were performed in an air thermostat which could be kept at a very constant temperature. The eyepiece of the bicolorimeter projected through a special opening in the thermostat, which was also provided with two small sliding doors for the adjustment of the wedges. The saline solution and

the bicolorimeter with the wedges were allowed to come to that temperature before the readings were made. The sample at room temperature was added to the saline in the same manner as above. The change in temperature due to the addition of 0.1 cc. of plasma at room temperature to the saline at 38° was very slight. To obtain the colorimetric value from the scale reading of the bicolorimeter account must be taken of the fact that the phosphate standards used for calibration become acid by 0.03 pH if the temperature is changed from 20° to 38°. Separate wedges were calibrated at 38° for use at this temperature. The color readings both at room temperature and at 38° were performed with duplicate samples. In order that the colorimetric determinations should be unbiased, the electrometric calculations were made at quite infrequent intervals.

The electrometric values were obtained by use of a modified Clark hydrogen electrode vessel of 2 cc. capacity constructed of Pyrex glass, with a thermometer opening directly into the chamber as recommended by Cullen (11). All determinations were performed in an air bath at 38°. At the beginning of each set of determinations 0.1 N HCl was placed in the electrode vessel and the resulting potential measured. From this the "*e*" of the calomel cell was calculated, a value of pH 1.085 being assigned to the HCl at 38°. The HCl was then removed and, after the vessel was washed with distilled water, the potential of a M/15 phosphate mixture of pH 7.4 was determined. When the calculated voltage for this was obtained, the phosphate was washed out with distilled water and the sample to be analyzed introduced. Since the CO_2 content was determined in every case, the approximate CO_2 tension to be introduced into the vessel could be estimated with the use of the colorimetric pH value. With the introduction of this approximate CO_2 tension into the electrode vessel constant voltages were usually obtained after one refill. All the potential measurements were corrected to 760 mm. of dry hydrogen.

Comparison of the Colorimetric pH at 20° and 38° with Electrometric pH at 38°.

In Table II are presented the results of a comparative study of colorimetric pH values obtained both at room temperature and at 38° with the true pH as determined electrometrically at

TABLE II.

Comparison of Electrometric and Colorimetric pH Values of Human Plasma.

Case No.	Age.	CO_2 content, vols. per cent.	pH_e 38°.	Corrections.		pH_c 20° − pH_c 38°.	Case No.	Age.	CO_2 content, vols. per cent.	pH_e 38°.	Corrections.		pH_c 20° − pH_c 38°.
				C	H						C	H	
				pH_c 20° − pH_e 38°.	pH_c 38° − pH_e 38°.						pH_c 20° − pH_e 38°.	pH_c 38° − pH_e 38°.	
1	33	81.5	7.54	0.22	0.01	0.21	29	54	65.5	7.41	0.18	0.00	0.18
2	36	77.7	7.44	0.28	0.10	0.20	30	36	65.5	7.41	0.26		
3	54	77.3	7.43	0.29			31	36	65.5	7.43	0.22		
4	35	75.8	7.48	0.18	−0.02	0.20	32	36	65.5	7.41	0.20	0.05	0.15
5	33	74.9	7.51	0.28			33	30	65.5	7.49	0.20		
6	48	74.0	7.50*			0.22	34	36	64.5	7.46	0.22		
7	34	74.0	7.46	0.27	0.04	0.23	35	61	64.5	7.46	0.18		
8	20	74.0	7.44	0.20			36	39	64.5	7.34*			0.21
9	55	73.0	7.49	0.24			37	7	63.6	7.45	0.23		
10	44	72.1	7.45	0.21	−0.02	0.23	38	18	63.6	7.41	0.20	−0.01	0.21
11	39	71.8	7.46	0.21			39	29	63.5	7.39	0.19		
12	35	71.1	7.39*			0.22	40	28	63.6	7.37	0.24		
13	53	71.1	7.37	0.23	0.11	0.12	41	36	63.5	7.43	0.19		
14	33	68.8	7.47*			0.23	42	20	62.6	7.36	0.21		
15	35	68.3	7.42	0.22	0.01	0.21	43	40	62.6	7.45	0.18	−0.03	0.21
16	34	68.3	7.41	0.26	−0.01	0.27	44	33	62.6	7.46	0.14	−0.07	0.21
17	52	68.3	7.46	0.21	0.04	0.17	45	11	61.7	7.48	0.19		
18	28	68.3	7.46	0.26	0.01	0.25	46	9	61.7	7.42	0.22		
19	36	67.3	7.39	0.19			47	53	60.7	7.45	0.22	0.02	0.20
20	27	67.3	7.46	0.19	0.01	0.18	48	22	60.7	7.39	0.28	0.03	0.25
21	28	66.9	7.44	0.19	−0.02	0.21	49	22	60.7	7.44	0.27	0.03	0.24
22	22	66.4	7.43	0.23	0.06	0.17	50	46	59.8	7.45	0.23		
23	36	66.4	7.50*			0.13	51	48	59.8	7.39	0.25	0.00	0.25
24	25	66.4	7.41	0.20	0.01	0.19	52	44	59.8	7.40	0.26	0.02	0.24
25	26	66.4	7.50*			0.13	53	58	59.8	7.39	0.24		
26	55	65.5	7.47	0.20			54	50	59.8	7.43	0.21	0.04	0.17
27	54	65.5	7.39	0.24	0.00	0.24	55	37	59.8	7.50	0.20		
28	54	65.5	7.34	0.24	0.03	0.21	56	54	58.9	7.38	0.24		

* The pH values marked with an asterisk were determined colorimetrically at 38°. In this and Tables III and IV the electrometric pH is designated pH_e and the colorimetric pH, pH_c. The pH_c 20° was corrected to 20° by adding 0.01 pH for each degree the room temperature was above 20°. The figures for the pH_c 20° and the pH_c 38° are omitted from the table, except the pH_c 38° in a few cases, since they may be calculated from the corrections and the pH_e 38°.

TABLE II—*Concluded.*

Case No.	Age.	CO_2 content, vols. per cent.	pH_e 38°.	Corrections. C pH_c 20° − pH_e 38°.	Corrections. H pH_c 38° − pH_e 38°.	pH_c 20° − pH_c 38°.	Case No.	Age.	CO_2 content, vols. per cent.	pH_e 38°.	Corrections. C pH_c 20° − pH_e 38°.	Corrections. H pH_c 38° − pH_e 38°.	pH_c 20° − pH_c 38°.
57	30	58.9	7.33*			0.18	88	4	54.1	7.32	0.15		
58	50	58.9	7.43	0.21	−0.01	0.22	89	50	54.1	7.44	0.26		
59	1	58.8	7.50	0.22			90	22	54.1	7.39	0.23	−0.01	0.24
60	30	57.9	7.50	0.23			91	51	54.1	7.39	0.20	−0.01	0.21
61	35	57.9	7.41	0.22	0.02	0.20	92	71	54.1	7.40	0.22		
62	59	57.9	7.39	0.28	0.11	0.17	93	33	53.2	7.34	0.27	0.02	0.25
63	32	57.9	7.39	0.19			94	44	53.2	7.36	0.26	0.02	0.24
64	50	57.9	7.41	0.25	0.06	0.19	95	61	53.2	7.46	0.19	0.00	0.19
65	33	57.9	7.43	0.30	0.02	0.28	96	61	53.2	7.44	0.19		
66	70	57.9	7.41	0.22			97	41	53.2	7.36	0.28	0.06	0.22
67	67	57.9	7.45*			0.22	98	36	53.2	7.44	0.23		
68		57.0	7.44*			0.24	99	7	53.1	7.38	0.22		
69	55	57.0	7.41	0.23			100	36	52.2	7.39	0.25		
70	53	57.0	7.44	0.20			101	55	52.2	7.33	0.25		
71	70	57.0	7.43	0.21			102	53	51.3	7.41	0.21		
72	65	57.0	7.41	0.24			103	73	50.4	7.40	0.20	0.05	0.15
73	70	57.0	7.44	0.22			104	33	50.4	7.41	0.25		
74	70	57.0	7.43	0.23			105	36	50.4	7.45	0.22		
75	6	57.0	7.46	0.24			106	26	50.4	7.42	0.24		
76	4	56.4	7.45	0.22			107	5	49.4	7.44	0.21		
77	21	56.0	7.52	0.24			108	48	49.4	7.40	0.26		
78	46	56.0	7.40	0.22	0.06	0.16	109	39	49.4	7.36	0.23		
79	50	56.0	7.45	0.23			110	2	47.2	7.36	0.17		
80	33	56.0	7.46*			0.19	111	46	45.7	7.38	0.23		
81	33	56.0	7.37*			0.18	112	½	44.7	7.36	0.20		
82	62	56.0	7.43	0.19			113	21	43.1	7.40	0.24		
83	29	55.1	7.40	0.24			114		40.9	7.40	0.26		
84	48	55.1	7.41	0.29	0.01	0.28							
85	36	54.8	7.48	0.21			Average.........				0.224	0.02	0.21
86	37	54.1	7.48	0.20			Maximum.......				0.30	0.11	0.28
87	6	54.1	7.44	0.17			Minimum........				0.14	−0.07	0.12

38° on 114 separated plasma samples. Inasmuch as the sex and diagnoses of the different cases did not seem to have any important bearing on the findings they have been omitted from Table II, as have the figures for the plasma chloride determined in 100 samples

and the plasma protein determined in 51 samples. The average C correction for 103 samples was 0.224, with maximum deviations from 0.14 to 0.30 pH. The value of 0.22 being assumed to be the average correction factor, 59 per cent of the values come within ±0.02 pH of this value, 74 per cent within ±0.03 pH, and 85 per cent within ±0.04 pH. In his original paper Cullen (1) states, "in following the condition of a patient, for example, under alkali therapy, the proposed method can be used with an accuracy of at least ±0.04 pH and probably with an accuracy of ±0.02. Whether or not extreme pathological conditions change the correction remains to be determined." The above findings agree very well with this statement.

TABLE III.

Comparison of Electrometric and Colorimetric pH Values of Dog Plasma.

Dog No.	pH_e 38°.	pH_c 20° (corrected).	$pH_c - pH_e$.	CO_2 content, vols. per cent.
1	7.38	7.67	0.29	43.0
	7.41	7.66	0.25	40.9
	7.39	7.64	0.25	38.1
	7.29	7.56	0.27	33.4
2	7.36	7.79	0.43	44.7
	7.39	7.69	0.30	43.8
	7.44	7.73	0.29	50.4
	7.43	7.74	0.31	40.9
	7.36	7.63	0.27	50.4
	7.35	7.68	0.33	34.3

The H correction on forty-one samples of plasma gives an average value of 0.02 pH, with maximum deviations of −0.07 to 0.11 pH. However, 59 per cent of these values fall within ±0.02 pH of having a zero correction. These findings confirm the observations of Hastings and Sendroy (6) "that colorimetric and electrometric determinations of the pH made on the same sample of blood agree to within 0.02 pH when both determinations are made at the same temperature."

The average value for the colorimetric readings of 51 samples of plasma corrected to 20°, minus the observed colorimetric value of 38°, is 0.21 pH. Since both colorimetric determinations were carried out with exactly the same technique, with the exception of

the temperature, the difference may be ascribed solely to differences in temperature. The average value found, 0.21 pH, is very close to the C correction. Attention has already been called to the greater difficulty of preventing loss of CO_2 in carrying out the colorimetric estimation at 38°. When this fact is taken into account it would seem that the agreement is within the limit of experimental error.

In Table III are presented the plasma pH determinations on two dogs, both before and after parathyroidectomy, the samples being taken on successive days. The average C correction for these ten determinations is definitely higher than the value for human plasma; namely, 0.30 pH.

DISCUSSION.

If the rather restricted groups of cases studied are kept in mind, it is felt that fair agreement between the two methods was obtained. Absolute agreement is not to be expected for, in spite of the precautions observed, there are factors such as the effect of dilution, changes in pH due to temperature, changes due to loss of CO_2, variation in the protein concentration, depth of color in the sample, and the actual matching of the indicator colors, which may possibly cause a discrepancy. We are inclined to believe that one reason why some workers have not obtained the most satisfactory results with the Cullen method is that, when test-tubes are employed, it is not so readily possible to match the color of the unknown against the standard as with the aid of the bicolorimeter. The greatest care must be exercised in taking the blood and separating the plasma, as a slight hemolysis influences the colorimetric reading. Bile, if present in any appreciable amount, invalidates the colorimetric pH value, despite the fact that with the aid of the yellow wedge of the bicolorimeter fairly satisfactory color matches may be obtained. For the present, however, where absolute values are desired reliance probably should be placed upon the electrometric or gasometric methods.

From the data presented in Table II there does not seem to be any apparent reason for the variations in the C corrections observed, nor for the higher value observed for the C correction in dog plasma. 51 protein determinations were made but from the figures, not included in Table II, there is no indication that a

change in protein concentration is a factor. Marrack and Smith (12) considered this point and found that the Cullen correction did not change with abnormalities in plasma proteins, and Hastings and Sendroy (6) concluded that at a 21-fold dilution the protein error is negligible. Marrack and Thacker (13), however, find that the protein effect on the indicator is not negligible at a 21-fold dilution and that the pH of buffer solutions to which plasma is added appears 0.05 to 0.07 pH too low when the protein concentration of the plasma is normal. Although changes in the salt concentration of urine may be sufficient to influence the pH values as determined colorimetrically (10), it is not believed that such changes in the blood are ever of sufficient magnitude to introduce a significant error.

It is quite conceivable, when one observes the dilution curve Cullen (1) obtained upon diluting serum with a saline solution of pH 7.4 to 7.5, that the plateau of the curve might be reached at varying dilutions depending upon the pH of the diluent in respect to the pH of the serum. That is, a serum of high pH diluted with a saline of pH 7.5 might have a quite different curve from a serum of a very low pH diluted with the same saline. One might expect greater deviation at the higher dilutions, although with lower dilution this may be minimized. With this in view various saline indicator solutions were prepared having such an indicator concentration that with the addition of the plasma, the final concentration of indicator was the same as in the wedges. The saline solutions were set at two pH levels; one series at a pH 7.5 to 7.6 and the other series at pH 7.25 to 7.35. As can be seen from Table IV, quite contrary to the effect of dilution observed by Cullen (1), the change of pH on dilution is very slight. One must conclude as did Hastings and Sendroy (6), "that 21-fold dilution *per se*, with an isohydrionic, isotonic NaCl solution has no measurable effect upon the pH of the solution." Small errors of the magnitude of 0.03 pH may be introduced, however, if the pH of the diluent is considerably removed from the pH of the plasma.

The colorimetric estimations at room temperature were made at about 25°. It is apparent that the application of a correction factor of 0.01 pH per degree, when the room temperature is above 20°, served very well in the samples here reported. This being the case, it is recommended that the colorimetric estimations be made

at room temperature rather than at 38°, since a satisfactory air thermostat is quite an expensive instrument to construct and the

TABLE IV.

Effect of Dilution with Saline Solutions Adjusted to Different pH Levels on pH of Plasma.

Sample No.	pH_s 38°.	pH of saline diluent.	Colorimetric pH corrected to 20°.			
			Dilution 1:21.	Dilution 1:15.	Dilution 1:10.	Dilution 1:5.
1	7.52	7.55	7.76			
		7.3	7.71	7.74	7.77	7.75
2	7.50	7.55	7.73			
		7.3	7.71	7.71	7.73	7.72
3	7.50	7.55	7.72	7.74	7.73	7.74
		7.3	7.68			
4	7.49	7.55	7.69			
		7.3	7.66	7.66	7.69	7.70
5	7.48	7.55	7.68			
		7.3	7.67	7.66	7.66	7.71
6	7.48	7.55	7.69	7.69	7.72	7.65
		7.3	7.65			
7	7.48	7.55	7.67			
		7.3	7.63			
8	7.46	7.55	7.70			
		7.3	7.67			
9	7.45	7.55	7.67			
		7.3	7.62	7.61	7.66	7.63
10	7.44	7.55	7.67			
		7.3	7.63	7.66	7.66	7.62
11	7.44	7.55	7.61	7.64	7.69	7.61
		7.3	7.61			
12	7.44	7.55	7.65	7.66	7.64	7.61
		7.3	7.64			
13	7.36	7.55	7.53	7.52	7.52	7.50
		7.3	7.50			
14	7.36	7.55	7.56			
		7.3	7.53			
15		7.55	7.65	7.65	7.68	
		7.3	7.62	7.61	7.63	7.61
16		7.55	7.68	7.67	7.65	
		7.3	7.65	7.60	7.61	7.62

manipulation of the bicolorimeter in it is somewhat less convenient. Furthermore it was found that the pH of the plasma under oil

became alkaline quite rapidly when heated to 38°. On this account the 0.1 cc. sample was not heated to 38° before it was introduced into the saline in the cup which was kept at 38°.

If the bicolorimeter is used at room temperature, readings of duplicate samples may be quickly obtained. As a rule when pH estimations are to be made, the CO_2 content will likewise be determined. For this reason there are advantages in using plasma. When, however, the quantity of blood is limited the method of Hawkins (14) may be slightly modified to adapt it to the bicolorimeter. 0.2 cc. of whole blood is added to 2 cc. of saline-indicator solution of pH 7.5 to 7.6 under oil in a special Pyrex tube with a bulb of about 2.2 cc. capacity, such as was formerly employed to separate the plasma (7). The mixture is stirred with the needle of the syringe and then centrifuged for about 10 minutes. A small amount of oil is placed in the cup of the bicolorimeter and the plasma-saline mixture separated from the cells with the aid of a syringe and transferred to the cup. The colors are then matched in the usual manner.

Several acid-base balance studies are being carried out in this laboratory in which use is being made of the colorimetric method. The values obtained are being compared with electrometric determinations in order to see whether further light may be thrown upon the apparent variations in the C correction. Consideration is being given as to the reason for the different C corrections necessary for dog and human plasma. In agreement with Hastings and Sendroy (6), it is felt that the deviations may be accounted for as a result of the change in pH due to temperature, depending upon the character and concentration of the proteins present.

SUMMARY.

Comparison has been made of the electrometric and colorimetric pH values of 114 samples of human blood plasma obtained from hospital patients.

Electrometric determinations of the pH at 38° and colorimetric determinations at room temperature have been made on 103 samples of blood plasma. The Cullen colorimetric method was employed, the color comparisons being made with the bicolorimeter essentially as described by Myers, Schmitz, and Booher. Very good agreement between the two methods was obtained, as

is indicated by the fact that, when the C correction 0.22 pH was used, 59 per cent of the colorimetric values were within ±0.02 pH, 74 per cent within ±0.03 pH, and 85 per cent within ±0.04 pH of the correct value.

The C correction for ten samples of dog plasma averages 0.30 pH.

The colorimetric pH at 38° was compared with the electrometric pH at 38° on forty-one samples of plasma. The H correction averaged +0.02 pH and 59 per cent of the colorimetric values were within ±0.02 pH of the true pH.

Subtracting the colorimetric pH obtained at 38° from the colorimetric pH corrected to 20° gave an average of 0.21 pH for 51 samples, which would indicate that the major part of the C correction is for the temperature change.

Since the pH of the plasma does not change appreciably on dilution when the pH of the saline diluent is properly adjusted, it is felt that possible variations in the C correction may result only as the temperature change is influenced by the character and concentration of the protein present.

BIBLIOGRAPHY.

1. Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 501.
2. Austin, J. H., Stadie, W. C., and Robinson, H. W., *J. Biol. Chem.*, 1925, lxvi, 505.
3. Hastings, A. B., Neill, J. M., Morgan, H. J., and Binger, C. A. L., *J. Clin. Inv.*, 1924–25, i, 25.
4. Cullen, G. E., Keeler, H. R., and Robinson, H. W., *J. Biol. Chem.*, 1925, lxvi, 301.
5. Bennett, M. A., *J. Biol. Chem.*, 1926, lxix, 697.
6. Hastings, A. B., and Sendroy, J., Jr., *J. Biol. Chem.*, 1924, lxi, 695.
7. Myers, V. C., Schmitz, H. W., and Booher, L. E., *J. Biol. Chem.*, 1923, lvii, 209.
8. Myers, V. C., *J. Biol. Chem.*, 1922, liv, 675.
9. Gatewood, W. E., Gaebler, O. H., Muntwyler, E., and Myers, V. C., *Arch. Int. Med.*, in press.
10. Myers, V. C., and Muntwyler, E., *J. Biol. Chem.*, 1928, lxxviii, 225.
11. Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 521.
12. Marrack, T., and Smith, F. C., *Brit. J. Exp. Path.*, 1924, v, 13.
13. Marrack, T., and Thacker, G., *Brit. J. Exp. Path.*, 1926, vii, 265.
14. Hawkins, J. A., *J. Biol. Chem.*, 1923, lvii, 493.

THE EFFECT OF INSULIN ON PROTEIN METABOLISM.*

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It was demonstrated recently by Luck, Morrison, and Wilbur (1) that insulin hypoglycemia in the normal animal is accompanied by a significant reduction in the amino nitrogen content of the blood. The problem, quite an obvious one, which then arose, was to ascertain the cause of this decrease and to determine the fate of the amino acids which disappeared.

Three fairly plausible explanations presented themselves. It seemed not improbable that just as glyconeogenesis accounts under certain conditions for some of the glucose which disappears from the blood of the insulin-treated animal, so a transient increase in the rate of protein synthesis, which might be presumed to occur, would be attended almost necessarily by a decreased concentration of amino acids in the blood. Were protein anabolism thus increased and accompanied by a corresponding depression in catabolism, a decrease in the rate of urea formation might be anticipated.[1] On the other hand, the decrease in amino nitrogen might be attributed to an increase in the rate of catabolism of the glucogenetic amino acids, without a compensatory increase in the rate of protein hydrolysis. Were this hypothesis true, a lowered aminoacidemia and an increased rate of urea formation would ensue. Finally, it appeared possible that the lowered concentration of amino acids in blood might be due to a migration of these substances from the blood to the tissues.

The choosing of the more acceptable of these hypotheses in-

* Part of the material in this paper is drawn from the thesis submitted by Veon Carter Kiech in candidacy for the degree of Master of Arts in Stanford University.

[1] The formation of "protein sugar" or of hypothetical amino acid-glucose compounds may be regarded as similar in kind, in the sense that a reduced rate of urea formation would follow.

volves only an examination of the effect of insulin on the amino acid content of various tissues and on urea formation. In view of the conflicts contained in the available evidence (2–14) on this latter point, it occurred to us that the problem might be examined profitably by determining not merely the urinary or blood urea but rather the total amount of that substance formed by the animal within given periods of time. With this end in mind we entered upon the experiments described.

Method.

Albino rats were used as the subjects of experiment. They were raised in our own colony and were maintained on the diet described elsewhere (15). 3 days before use the animals were placed on special diets of either high or low protein content. For the former we employed a mixture of the following composition: casein 87 per cent, yeast 8 per cent, alfalfa 5 per cent. The low protein diet consisted of butter 49 per cent, starch 49 per cent, sodium chloride 2 per cent. In order to insure the freedom from nitrogenous food of the stomach and small intestine, the animals on high protein diets were fasted for the last 12 to 15 hours.

For the purpose of this investigation, the animals were used in groups of six. Each group was divided into two sets of three animals each, referred to hereafter as the controls and the insulin animals. The former received subcutaneous injections of 1 per cent sodium chloride, while the latter received insulin, diluted to a strength of 1 unit per cc. with 1 per cent sodium chloride. Whenever an insulin animal received an injection of insulin, its corresponding control received an equal volume of 1 per cent sodium chloride.

The injections, two or three in number, were distributed over a preliminary period of 4 hours. At the end of this time the animal, supported by a coarse wire screen, was placed over an 8 inch porcelain basin. A trace of mercuric chloride was added to the basin to prevent ureolysis. The bladder was first emptied and the urine discarded.[2] The purpose of this preliminary period was

[2] It was important for our purposes that the urine previously formed be voided. This was accomplished by giving the animal a breath or two of ether,—in quantity much below an anesthetic dose. Emptying of the bladder was prompt and complete. We are indebted to Dr. T. Addis for this technique.

to rid the animal of preformed urea. We were interested only in the insulin phase of urea formation. The last injection was designed to maintain the animal under the influence of insulin during the period of urine collection.

In none of the high protein animals did convulsions intervene. We encountered much difficulty, however, in properly adjusting the dosage for the low protein animals owing to their relatively

TABLE I.

Preliminary Experiments.

Rat No.	Weight.	Time after injection.	Mg. per 100 gm. tissue.		Remarks.
			Urea.	Amino acid N.	
	gm.	*hrs.*			
1	150	0	56.4	42.9	One injection of sodium chloride. Male rats. Low protein diet.
2	155	1	62.9	41.2	
3	152	2	69.3	47.0	
4	160	4	67.9	46.0	
5	154	6	80.0	49.6	
1	152	2	42.9	44.1	One injection of sodium chloride. Female rats. Low protein diet.
2	130	2	45.0	41.6	
3	135	3	45.7		
4	146	3	48.6	42.7	
5	151	4		42.5	
6	146	4		42.7	
1	138	0	51.5	40.9	One injection of insulin. Female rats. Low protein diet.
2	123	1	57.2	44.2	
3	135	2	58.0	39.2	
4	138	4	66.5	42.8	
5	128	6	{76.5	41.2	
			77.0	41.2	

low tolerance to insulin. Most of these animals experienced convulsions.

The animals were killed in pairs, at intervals of 1, 2, and 4 hours, after the collection of the excreta was commenced. Each pair consisted of one control and one insulin animal. Although these intervals, relative to the time of commencement, were closely observed, it is apparent that the absolute times of first injection had to be adjusted to suit the convenience of the experimenter.

TABLE II.

Low Protein Diets.

Time of killing insulin animals.	Insulin animals.			Control animals.			Remarks.
	Weight.	Mg. per 100 gm. tissue. Urea.	Mg. per 100 gm. tissue. Amino acid N.	Weight.	Mg. per 100 gm. tissue. Urea.	Mg. per 100 gm. tissue. Amino acid N.	
a.m.	*gm.*			*gm.*			
10.30	143	65.5	45.1				Females.
p.m.							Insulin injections.
2.00	150	70.5	41.1	149	54.3	50.5	4 units per kg. 8.00 a.m. 1 unit " " 1.00 p.m.
2.40	154	72.9	41.6	123	67.2	45.9	0* time, 1.00 p.m.
2.30	183	78.0	50.0	175	58.0	49.5	Males. Insulin injections.
3.30	180	70.0	40.9	182	53.0	42.0	2 units per kg. 8.00 a.m. 2 " " " 10.00 "
4.00	152	63.0	35.7				1 unit " " 1.00 p.m. 2 units " " 1.30 " 0 time, 1.30 p.m.
2.30	143	58.0	38.6	142	Lost.	Lost.	Females. Insulin injections.
3.30	130	70.5	49.3	134	70.0	51.8	3 units per kg., 8.00 a.m. 2 " " " 10.00 "
5.30	142	80.5	41.8	135	67.0	48.7	0 time, 1.30 p.m.
1.10		Lost.	Lost.	130	53.0	45.4	Females. Insulin injections.
2.10	124	58.5	42.0	124	54.3	45.7	3 units per kg., 8.10 a.m. 1 unit " " 10.10 "
4.10	130	67.1	39.9	126	57.2	44.5	1 " " " 12.10 p.m. 0 time, 12.10 p.m.
4.00	138	76.5	33.2	134	65.0	41.9	Males. Insulin injections.
4.00	147	85.0	37.8	142	65.5	40.8	3 units per kg., 8.00 a.m. 2 " " " 10.00 "
4.00	127	Lost.	Lost.	136	67.0		1 unit " " 12.00 p.m. 0 time, 12.00 p.m.
Averages..		70.5	41.3		60.9	46.1	

* 0 time is the time at which collection of the urine was commenced.

It was necessary that a 30 minute interval separate the killing of successive animals in order to provide ample time for mincing and freezing the carcass, weighing the sample, and preparing the protein-free extract.

TABLE III.

High Protein Diets.

Time of killing insulin animals.	Insulin animals.			Control animals.			Remarks.
		Mg. per 100 gm. tissue.			Mg. per 100 gm. tissue.		
	Weight.	Urea.	Amino acid N.	Weight.	Urea.	Amino acid N.	
p.m.	*gm.*			*gm.*			
							Males.
							Insulin injections.
1.10	125	97.2	40.9	156	92.2	50.1	3 units per kg., 8.10 a.m.
							2 " " " 10.10 "
2.10	143	90.0	50.7	141	100.7	50.4	1 unit " " 12.10 p.m.
4.10	128	143.5	43.1	124	112.2	49.0	0* time, 12.10 p.m.
1.45	117	103.2	48.5	113	95.0	50.3	Males.
							Insulin injections.
2.45	118	133.5	46.3	117	101.5	50.8	3 units per kg., 8.45 a.m.
							2 " " " 10.15 "
4.45	111	160.0	50.2	110	141.5	52.2	1 unit " " 12.45 p.m.
							0 time, 12.45 p.m.
2.15	113	103.9	47.9	114	95.7	53.5	Males.
							Insulin injections.
3.15	119	105.7	47.9	123	96.0	55.0	4 units per kg., 8.15 a.m.
							3 " " " 11.15 "
4.15	116	133.0	38.2	110	108.0	52.2	1 unit " " 1.15 p.m.
							0 time, 1.15 p.m.
Averages...		118.9	46.0		104.8	51.5	

* 0 time is the time at which collection of the urine was commenced.

The animal was killed by a blow and immediately run through a meat grinder. The mincings which were promptly treated with liquid air were received in the same basin which had served to collect the excreta of the animal. The subsequent steps in the

procedure have been described in the preceding paper (16). It will be seen that we have determined by this technique the total extent of urea formation and of total amino acid nitrogen (residual + excreted). It is also to be observed that by experimentation upon pairs of animals, of which one was a control and the other an insulin animal, we have limited very greatly the number of uncontrolled variables.

Results.

In Table I we have presented the results of several preliminary experiments, in which the routine procedure of the later work had not been adopted. They serve to demonstrate the measure of agreement between analyses of duplicate samples from the same animal, and between pairs of animals subjected to the same treatment. They serve also to demonstrate the rate of urea formation in the fasting animal,—roughly 4 mg. per hour per 100 gm. of body weight.

In Table II we have recorded the results of administering insulin to a number of rats on low protein diets. The animals were investigated in five groups of three pairs each. It will be observed that in every instance the urea formation of the insulin animal is greater than that of its corresponding control. It is also apparent that an inverse relationship exists with respect to the amino acid nitrogen, for in all cases but one the amino nitrogen value for an insulin animal is less than that of its corresponding control.

Of similar nature are the results which followed the administration of insulin to animals on high protein diets. The findings are summarized in Table III.

DISCUSSION.

We are drawn to conclude that under the conditions of these experiments insulin increases the rate of amino acid catabolism and inhibits the compensatory process of protein hydrolysis by which the amino acids are generated. These conclusions follow from the increased rate of urea formation here observed together with the associated decrease in the amino nitrogen content of the animal. On comparing the average increase in urea with the average decrease in amino acid nitrogen, it is to be observed (Table II) that a change of 9.6 mg. in the former (70.5 − 60.9) is asso-

ciated with a change of 4.8 mg. (46.1 − 41.3) in the latter. The ratio $\frac{9.6}{4.8}$ (which equals 2.0) approaches closely the ratio $\frac{60}{28}$ (*i.e.* 2.14) which may be calculated on the assumption that all of the extra urea is formed at the expense of the amino acid nitrogen which has disappeared. This indicates an almost complete inhibition in the compensatory process of amino acid formation. Likewise the data of Table III require the same interpretation. This fact is in rather striking contrast to the common observation that an increased rate of urea formation is associated with an increased amino nitrogen content.

Mention should also be made of these effects upon protein metabolism in relation to the changes in carbohydrate metabolism induced by insulin. It will be recalled that Bissinger and Lesser (17) in observations upon mice, and Best, Dale, Hoet, and Marks (18) using the technique of Burn and Dale (19) demonstrated that all of the glucose utilized was either oxidized or converted into glycogen. If, however, the increased rate of protein catabolism here observed is due to an increased rate of glucogenesis from amino acids, it follows that the calculated quantity of reducing sugar which disappears from an animal as a result of insulin administration must be increased by a second fraction of considerable magnitude. Assuming that 40 per cent of the extra protein catabolized in a 4 hour period participated in glucogenesis, the extra glucose formed would be 15 to 20 mg. per 100 mg. of body weight or 200 to 250 mg. per 100 cc. of blood. It is therefore probable that the quantitative estimates which have been made of the fate of the blood sugar in insulin-treated animals are in need of correction.

We now propose to examine the hypothesis that the increased rate of protein catabolism is due to glucogenesis, and to determine whether the effects are secondary to the accompanying hypoglycemia.

SUMMARY.

1. The effect of insulin on the protein metabolism of rats was investigated by analyzing the entire carcass for urea and amino acid nitrogen within 1 to 4 hours after the commencement of the experimental period. The values obtained were compared with

those of control animals which received injections of sodium chloride.

2. Marked increases in the rate of urea formation were observed.

3. The amino acid nitrogen content of the whole animal decreased. The average decrease observed was approximately equal to the average increase in urea nitrogen.

4. The relationship between these results and the attendant changes in carbohydrate metabolism is discussed.

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BIBLIOGRAPHY.

1. Luck, J. M., Morrison, G., and Wilbur, L. F., *J. Biol. Chem.*, 1928, lxxvii, 151.
2. Paulesco, N. C., *Arch. internat. physiol.*, 1921, xvii, 85.
3. Janney, N. W., and Shapiro, I., *Arch. Int. Med.*, 1926, xxxviii, 96.
4. Tashiro, K., *Tohoku J. Exp. Med.*, 1926, vii, 268.
5. Mazzocco, P., and Morera, V., *Compt. rend. Soc. biol.*, 1924, xci, 30.
6. Blatherwick, N. R., Bell, M., and Hill, E., *J. Biol. Chem.*, 1924, lxi, 241.
7. Allan, F. N., and Macleod, J. J. R., *Tr. Roy. Soc. Canada*, 1923, xvii, sect. 5, 47.
8. Kudrjawzewa, A., *Z. ges. exp. Med.*, 1924, xliv, 313.
9. Deuel, H. J., Jr., and Chambers, W. H., *J. Biol. Chem.*, 1925, lxv, 7.
10. Nash, T. P., Jr., *J. Biol. Chem.*, 1923–24, lviii, 453; 1925, lxvi, 869.
11. Ringer, M., *J. Biol. Chem.*, 1923–24, lviii, 483.
12. Sokhey, S. S., and Allan, F. N., *Biochem. J.*, 1924, xviii, 1170.
13. Chambers, W. H., and Milhorat, A. T., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 170.
14. Labbé, H., *Presse méd.*, 1924, xxxii, 542.
15. Luck, J. M., *J. Biol. Chem.*, 1928, lxxvii, 13.
16. Kiech, V. C., and Luck, J. M., *J. Biol. Chem.*, 1928, lxxvii, 723.
17. Bissinger, E., and Lesser, E. J., *Biochem. Z.*, 1926, clxviii, 398.
18. Best, C. H., Dale, H. H., Hoet, J. P., and Marks, H. P., *Proc. Roy. Soc. London, Series B*, 1926, c, 55.
19. Burn, J. H., and Dale, H. H., *J. Physiol.*, 1924, lix, 164.

THE TRANSPORT NUMBERS OF FIBRIN IN SOLUTIONS OF DILUTE ACIDS AND ALKALIES.*

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INTRODUCTION.

In recent years considerable use has been made of electrical transference measurements as an aid in determining the structure of colloidal solutions (1). In this way the writer and Schmidt (2, 3) were able to show that in solutions of casein in dilute alkali hydroxides the current is carried by the cations of the alkali and casein anions quite as in any simple salt solution and the mobility of the casein anion was calculated to have about the same value as lithium ion at 25°. With solutions of casein in the alkali earth hydroxides, it was found that a considerable portion of the cations were bound to the casein to give negatively charged complex ions. It is desirable to extend such studies to other proteins to find how general such a behavior is of proteins as a class.

After some preliminary considerations, fibrin was chosen as a suitable protein for further investigation. Also, since casein is not sufficiently soluble in acid solutions to make studies on the acid side of the isoelectric point and fibrin is sufficiently soluble, transference measurements were made on acid solutions of fibrin. It would also have been of interest to study the behavior of fibrin in solutions of bivalent acids and bases. However, as the fibrin was found not to dissolve in the alkali earth hydroxides and the bivalent acids tested, it was not possible to make electrochemical studies on such solutions.

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EXPERIMENTAL.

The experimental procedure for the transference measurements are the same as those described for casein (2). Determinations were always carried out in duplicate. The cells used were made of Pyrex glass, with 10 mm. bore stop-cocks between the middle and end compartments. Each of the three compartments, cathode, middle, and anode, was approximately of 75 cc. capacity. In the alkali solutions a platinum wire was used as anode and a lead wire covered with lead peroxide for cathode. In the acid solutions, the platinum wire was used as the cathode and a silver wire for anode. A 220 or 110 volt source was used to give a current of from 2 to 10 milliamperes as desired. The amount of current passed through the cells was measured by silver titration coulometers. The measurements were carried out in an air bath, controlled by a thermoregulator, and a fan in the bath acted as a stirrer.

The analysis of fibrin was made by drying 10 gm. portions in porcelain crucibles at 105° to constant weight. The weight of the cation or anion in the solution was subtracted from the total. The amount of cation or anion was determined by titrating to the isoelectric point of the fibrin (point of maximum flocculation) 40 cc. portions of the solution, 0.025 N hydrochloric acid being used to titrate the alkaline solutions and 0.025 N NaOH the acid solutions. The titration procedure was the same as has been given for casein.

In preparing and purifying the fibrin used for the experiments, a number of innovations were introduced. The basis of the procedure for the preparation is that described by Bosworth (4). In all fibrin was prepared by three different methods in order to test the effect of differences in methods of preparation.

Methods of Preparation.

About a 50 gm. portion (on a dry weight basis) of freshly whipped beef fibrin was washed with cold water to free it from as much blood as possible. It was then ground in a fine cutting meat chopper and washed into a tall glass cylinder of 4 liters capacity. The ground fibrin was now washed with distilled water by decantation to free it from blood and soluble matter, until the

supernatant wash water was free of color. Up to this point the treatment is similar for all of the preparations. The fibrin was now washed into a 4 liter Erlenmeyer flask and for the alkali preparation, 50 cc. of 1 N NaOH were added to make the pH approximately 10. The flask was then heated on an electrically warmed water bath at from 60–70° until nearly all of the fibrin was dispersed. This usually took about 7 hours and the solution became colored a greenish brown as a result of the heating. The solution was now filtered, first through cheese-cloth to take out the large undissolved particles and then several times through cotton flannel cloth, with suction. The liquor was now poured into a 4 gallon crock and 1 N HCl slowly run in from a burette, the solution being vigorously stirred all the while with a motor-driven stirrer. The acid was run in until the point of maximum flocculation was reached, the process being controlled by withdrawing 5 cc. samples from time to time, adding an equal amount of distilled water, and noting the rapidity and completeness of settling. The finely divided flocculated fibrin was now again poured into a tall glass cylinder and washed with cold distilled water by decantation until free from chloride ion. The fibrin was then either washed with alcohol and ether and dried, or in some cases a little toluene was added to the thick aqueous suspension, which was then kept in a refrigerator, portions being drawn off for experiments as needed. Samples prepared in this way by alkali dispersion are designated in the data as B with a subscript numeral to indicate the number of the preparation.

Fibrin was prepared by dispersing in acid, with hydrochloric acid as the dispersing agent. The steps in the preparation are the same as for the alkali-dispersed fibrin. About 50 cc. of 1 N HCl were used to disperse approximately 50 gm. of fibrin. The temperature of heating was the same as given above and the flocculation was also carried out as described, 1 N NaOH being used to neutralize the HCl. Preparations made in this way are designated as A.

The A preparation of fibrin always gave turbid solutions, so to obtain fibrin intermediate in properties between the acid and alkali method of preparation, recourse was had to the following procedure.

The fibrin was first dispersed in HCl as described above, fil-

tered, neutralized with NaOH, and then washed by decantation. Then the fibrin was again introduced into a large Erlenmeyer flask, enough NaOH was added to make the solution pink to phenolphthalein, and the flask again heated on the water bath at about 65° for about 2 hours. The liquor was then again filtered, flocculated with HCl, and washed free from electrolytes by decantation. Fibrin prepared in this way is designated as C.

Properties of Fibrin Preparations.—All of the alkali-dispersed fibrin (B) preparations yielded very viscous solutions when dissolved in dilute hydroxide solutions. In acid solution they did not dissolve completely, becoming greatly swollen and viscous, but on centrifuging they were thrown down with the separation of a dilute fibrin-containing liquor. On this account it was not possible to make measurements of transport numbers in acid

TABLE I.

Preparation.	Isoelectric region.	N	Ash.
	pH	*per cent*	*per cent*
B_1	4.95–5.3	15.8	0.30
A_1	4.55–6.85	15.7	0.38
C_1	4.5 –6.75	15.4	0.37
C_3		14.8	0.23

solutions with B fibrin. The acid preparations A gave turbid solutions in both acid and alkali. When first made up the solutions were too viscous for use, but on standing overnight or warming at 50° for an hour or two the solutions became quite fluid. Fibrin (C) gave brownish-colored solutions with both acid and alkali which were quite homogeneous to the naked eye.

A comparison of the nitrogen content, ash content, and isoelectric region of some of the samples prepared by each of the different methods is given in Table I.

The isoelectric region was determined by flocculating solutions made up in NaOH and then adding various amounts of HCl, and also *vice versa*. The pH was determined with a 0.1 N calomel hydrogen electrode. It is to be noted that fibrin has no sharp isoelectric point, but instead a fairly broad zone, and the alkali treatment narrows the zone and shifts the isoelectric region considerably to the acid side.

Without doubt, some decomposition is produced in all of the treatments used in the preparation of the fibrin. Indeed, the odor of H_2S was noted during all of the preparation work. It is not claimed that these preparations are identical with native untreated fibrin nor that the measurements are identical with what would be obtained on native fibrin.

DISCUSSION.

The transport number data for fibrin in alkali solutions are given in Tables II to IV and for acid solutions in Tables V to X. The column headings are for the most part self-explanatory. B represents the amount of base in cc. of 0.1 N added for each gm. of fibrin in the alkali solutions and A represents the amount of 0.1 N acid added per gm. of fibrin in acid solutions. K is an electrochemical constant, the product of either B or A and Q, in which Q is the electrochemical equivalent of the fibrin and is expressed in terms of the gm. of fibrin deposited on either anode or cathode on the passage of 1 millifaraday of current. Since Q can be directly obtained by dividing K by B, it is unnecessary to include the values in the tables. Under "remarks" are given some of the observations noted on the solutions employed.

Electrochemical Equivalent.—In a previous communication (2) it was shown that the deposition of casein on the anode by an electric current, obeys a modification of Faraday's law of electrodeposition, the relationship found being

$$B \times Q = K \tag{1}$$

An examination of the values under K in the tables of data shows that this relationship also holds for fibrin solutions. However, the value of K is seen to be dependent on the method of preparation of the fibrin. In alkali solutions, B-prepared fibrin has the highest, C intermediate, and A the lowest value of K. In acid solutions, the order is reversed, A fibrin giving the highest value of K. As it was not possible to obtain acid solutions of B fibrin it is not possible to state whether the reversal is complete.

The maximum value that the constant K can obtain, with the units given, is 10.0. This is to be seen from the following considerations. If all the alkali added is in combination with the

TABLE II.

Transport Numbers of Fibrin Solutions in Sodium Hydroxide.

Temperature 25°.

Fibrin preparation.	Fibrin.	B	pH	K	T fibrin$^-$.	T Na$^+$	Remarks.
	per cent	*cc. 0.1 N*					
B_1	1.65	4.60	8.7	9.2	0.42	0.74*	
	1.65	4.60	8.7	9.3	0.44	0.60	
	1.62	3.47	7.6	9.2	0.72*	0.41	Solution very viscous, neighborhood of electrodes became decolorized.
	1.62	3.47	7.6	9.0	0.81*	0.44	
	1.83	4.15	8.3	9.4	0.50	0.65	Viscous solution, color at top of anode column lighter at end of experiment.
	1.83	4.15	8.3	9.5	0.55	0.57	
	1.64	6.80	10.0	8.4	0.44	0.31*	
	1.64	6.80	10.0	8.5	0.45	0.61	
B_2	1.32	5.20	9.35	9.4	0.82*	0.47	Solution very viscous.
	1.32	5.20	9.35	9.8	0.54	0.38	
Average............				9.2	0.475	0.52	
A_1	1.64	3.84	9.7	6.0	0.47	0.41	Solution turbid.
	1.64	3.84	9.7	6.0	0.61*	0.42	" "
	1.50	4.72	10.2	5.8	0.46	0.49	" "
	1.50	4.72	10.2	5.9	0.52	0.35	" "
	1.80	4.07	9.9	6.3	0.69*	0.49	" "
	1.80	4.07	9.9	6.3	0.52	0.53	" "
	1.99	6.00	10.5	5.8	0.51	0.61	" "
	1.99	6.00	10.5	6.0	0.50	0.52	" "
	1.88	6.20	10.6	5.6	0.42	0.43	" "
	1.88	6.20	10.6	5.7	0.50	0.47	" "
Average............				5.95	0.485	0.47	
C_2	1.82	5.50	9.6	7.9	0.45	0.48	
	1.82	5.50	9.6	7.8	0.45	0.38	
C_3	1.50	5.50	9.7	7.6	0.43	0.54	
	1.50	5.50	9.7	7.2	0.48	0.52	
	1.51	5.66	9.7	7.0	0.48	0.50	
	1.51	5.66	9.7	7.2	0.46	0.55	
Average............				7.45	0.46	0.50	

* Value not used in obtaining average.

fibrin and each unit of fibrin carries its proportionate share of the charge, then the fibrin in the solution has the same value in equivalents as the base added. Now, if we take as a numerical example a solution containing 4.0 cc. of 0.1 N alkali per gm. of protein, *i.e.* $B = 4.0$, then for each equivalent of base in the solution there corresponds the amount $\frac{10,000}{4} = 2500$ gm. of fibrin. In other words, the equivalent weight of the fibrin in the solution is 2500. On the passage of 1 millifaraday of current one-

TABLE III.

Transport Numbers of Fibrin Solutions in Potassium Hydroxide.

Temperature 25°.

Fibrin preparation.	Fibrin.	B	pH	K	T fibrin$^-$.	T K$^+$	Remarks.
	per cent	*cc. 0.1 N*					
A_1	2.09	6.40	10.6	5.5	0.38	0.57	Solution turbid.
	2.09	6.40	10.6	5.6	0.43	0.56	" "
	1.86	5.00	10.3	6.6	0.37	0.59	" "
	1.86	5.00	10.3	6.4	0.38	0.55	" "
	1.69	4.57		6.4	0.33		" "
	1.69	4.57		6.9	0.48*	0.35*	" "
Average				6.2			
C_1	1.76	5.74	10.1	8.1	0.37	0.53	
	1.76	5.74	10.1	8.1	0.38	0.62	
	1.66	5.95	10.3	7.9	0.36	0.62	
	1.66	5.95	10.3	7.9	0.37		
Average				8.0	0.375	0.58	

* Value not used in obtaining average.

thousandth of this, or 2.5 gm., should be deposited on the anode, *i.e.* Q would be 2.5. It follows then that K is $4.0 \times 2.5 = 10.0$, which is the maximum possible value. However, if a portion of the fibrin is not carrying its proportionate share of the charge, then the equivalent weight calculated from the base in the solution will be high, since the equivalent weight of the protein varies inversely as B and consequently K will be lower than 10.0. The same considerations hold for solutions of protein in acid.

Since the fibrin solutions all give values of K less than 10.0, it must be concluded (1) that there is not an equal distribution of charges on the protein units and (2) that the tendency to discharge on the passage of the current varies among the different charged units present. An alternative explanation would be that uncharged as well as charged fibrin is present in the solution and only the charged fibrin is deposited on the electrode. The amount of

TABLE IV.

Transport Numbers of Fibrin Solutions in Lithium Hydroxide.

Temperature 25°.

Fibrin preparation.	Fibrin.	B	pH	K	T fibrin$^-$.	T Li$^+$	Remarks.
	per cent	*cc. 0.1 N*					
A_2	2.39	3.55	9.9	5.7	0.52	0.49	Acid step in preparation of C_2, solutions colored brown.
	2.39	3.55	9.9	5.3	0.46	0.48	
	2.38	3.70	10.0	5.6	0.53	0.43	
	2.38	3.70	10.0	5.3	0.53	0.47	
Average				5.5			
C_2	1.70	5.90	9.6	7.5	0.51	0.44	
	1.70	5.90	9.6	7.5	0.52	0.40	
	1.80	5.70	9.7	7.8	0.52	0.47	
	1.80	5.70	9.7	7.4	0.46	0.44	
	1.58	6.33	10.0	7.3	0.50	0.44	
	1.58	6.33	10.0	7.0	0.52	0.42	
	2.39	4.06	8.7	8.0	0.48	0.42	
	2.39	4.06	8.7	7.8	0.49	0.42	
	2.18	6.45	10.1	7.2	0.57	0.42	
	2.18	6.45	10.1	6.7	0.59	0.40	
Average				7.4	0.515	0.44	

the uncharged fibrin would be expected to be dependent on the treatment to which the fibrin is subjected. In alkali solutions, B, fibrin gives a K value of over 9.0 which is a close approach to the maximum of 10.0, C fibrin gives an average value of nearly 7.5, and A of 6.0. Evidently warming with alkali increases the capacity for combination with alkali as is shown by the increase in K from fibrin A $\rightarrow$ B $\rightarrow$ C.

Transport Numbers in Alkali Solutions.—Although the electro-

chemical constant of a fibrin sample depends on its method of preparation, the transport number depends only on the alkali in which it is dissolved. This is well illustrated by the results in NaOH solution given in Table II. Determinations were made

TABLE V.

Transport Numbers of Fibrin Solutions in Hydrochloric Acid.

Temperature 25°.

Fibrin preparation.	Fibrin.	*A*	pH	*K*	*T* fibrin+. Cathode.	*T* fibrin+. Anode.	*T* Cl−. Cathode.	*T* Cl−. Anode.	Remarks.
	per cent	*cc. 0.1 N*							
C₁	1.49	5.54	3.2	2.3*	0.36*		0.67		Solution viscous, deposit did not adhere to electrode.
	1.49	5.54	3.2	1.9*	0.51		0.60		
	2.23	4.87		5.7	0.46	0.43	0.46	0.62	Solution heated 4 hrs. at 55°.
	2.23	4.87		5.6	0.46	0.40	0.52	0.55	Solution turbid.
	2.12	3.85	3.5	5.9	0.46	0.39	0.47	0.62	AgCl did not stick to electrode completely.
	2.12	3.85	3.5	5.7	0.46	0.38	0.53	0.56	
Average				5.7					
A₂	2.79	2.34		7.3	0.63		0.33		Solution heated 4 hrs. at 60°.
	2.79	2.34		7.7	0.63		0.31		
	1.59	4.20		7.0	0.63		0.51		Solution not heated; very viscous.
	2.14	4.20		7.8	0.52		0.39		Solution heated 3 hrs. at 75°.
	2.14	4.20		7.8	0.48		0.60		
	1.69	5.10		6.7	0.46		0.49		
	1.69	5.10		6.4	0.49		0.56		
Average				7.2	0.51		0.50		

* Value not used in obtaining average.

on fibrin prepared by all three methods, yet the average of the fibrin transport number of each type of fibrin is within the limit of error of the method the same for all. The mobility of the negatively charged fibrin ion or micelle, it follows, is independent of the method of preparation. The sum of the transport numbers

of the fibrin and the respective cation of the solutions is seen from the tables to be approximately unity. The alkalinity of all the solutions employed was so low that the OH ion concentration as a factor in carrying the current can be neglected. Since the values of the fibrin and cation transport are determined by independent titration, it indicates that the only significant carriers

TABLE VI.

Transport Numbers of Fibrin Solutions in Hydrobromic Acid.

Temperature 25°.

Fibrin preparation.	Fibrin.	*A*	pH	*K*	*T* fibrin⁺. Cathode.	*T* fibrin⁺. Anode.	*T* Br⁻ Cathode.	*T* Br⁻ Anode.	Remarks.
	per cent	*cc. 0.1 N*							
C_3	1.43	4.88	3.3	4.9	0.51	0.50	0.31*	0.60	
	1.43	4.88	3.3	4.8	0.46	0.53	0.30*	0.64	
	1.67	5.30	3.1	4.8	0.52	0.49	0.40		
	1.67	5.30	3.1	4.6	0.51	0.48	0.37		
	2.07	4.05	3.5	5.4	0.50	0.47	0.44	0.64	
	2.07	4.05	3.5	5.2	0.51	0.49	0.39	0.66	
Average............				4.95		0.49		0.63	
A_2	1.89	4.80		7.0	0.42*		0.46		Heated 2 hrs. at 75°.
	1.89	4.80		6.9	0.41*		0.49		
	1.54	4.80		6.3	0.28*		0.48		Solution unheated; very viscous.
	1.54	4.80		6.2	0.27*		0.57		
Average............				6.6	0.50		0.45		

* Value not used in obtaining average.

of current in such a solution are protein and alkali ion. Due to the small quantity of alkali change in an experiment, the greatest error comes in the calculation of the cation transport numbers. In Table XI is given the mobility of the fibrin calculated from the averaged values of the transport numbers with each of the cations employed, by the formula

$$\Lambda^0 \text{ fibrin} = \frac{T \text{ fibrin}}{T \text{ cation}} \Lambda^0 \text{ cation} \qquad (2)$$

The values calculated indicate that the mobility of the fibrin, like that of casein, is independent of the cation and that these solutions are completely ionized in the sense that this term is used in the interionic attraction theory of electrolytes (5).

TABLE VII.

Transport Numbers of Fibrin Solutions in Nitric Acid.

Temperature 25°.

Fibrin preparation.	Fibrin.	A	pH	K	T fibrin+.	T NO_3^-	Remarks.
	per cent	*cc. 0.1 N*					
C_1	2.02	4.48		5.9	0.54	0.36	Solution heated 2 hrs. at 58°; turbid.
	2.02	4.48		5.9	0.54	0.34	
	2.18	4.32	3.4	6.3	0.60*	0.35	Solution heated 3 hrs. at 58°; turbid.
	2.18	4.32	3.4	6.2	0.53	0.42	
	2.17	4.30	3.4	6.3	0.56	0.33	
	2.17	4.30	3.4	6.2	0.57	0.33	
Average				6.1	0.55	0.36	

* Value not used in obtaining average.

TABLE VIII.

Transport Numbers of Fibrin Solutions in Formic Acid.

Temperature 25°.

Fibrin preparation.	Fibrin.	A	pH	K	T fibrin+.	T formate−.	Remarks.
	per cent	*cc. 0.1 N*					
C_2	1.51	9.40	3.5	5.0	1.46	0.38	Heated 1 hr. at 55°.
	1.51	9.40	3.5	5.2	1.30	0.30	
	1.91	6.70		5.1	1.09	0.35	Heated 1 hr. at 55°.
	1.91	6.70		4.8	1.15	0.35	
	2.13	5.90	3.45	4.1	1 35	0.28	Heated 1 hr. at 60°.
	2.13	5.90	3.45	4.8	1.35	0.38	
Average				4.8	1.28	0.34	

Transport Numbers of Fibrin in Solutions of Strong Acids.— Direct measurements of the transport numbers of a protein in acid solutions have heretofore not been made. Ferguson and France (6) and France and Moran (7) investigated the influence of gelatin on the transport numbers of sulfuric and hydrochloric

acids, using concentration cells with transference for this purpose. The theoretical basis for the formulas used by the above authors in calculating the transport numbers of their solutions containing gelatin has been criticized by Scatchard (8). Foregoing Scatchard's criticism, it was found, on successive additions of gelatin that the transport number of the H ions decreased and that the transport numbers of the sulfate and chloride ions increased, reaching almost a constant value on the addition of over 6 per cent gelatin to the cells, which consisted of 0.01 N and 0.1 N acid plus gelatin. Aside from this, a few quantitative measure-

TABLE IX.

Transport Numbers of Fibrin Solutions in Phosphoric Acid.

Temperature 25°.

Fibrin preparation.	Fibrin.	A	pH	K	T fibrin^{+}.	T $H_2PO_4^{-}$	Remarks.
	per cent	*cc. 0.1* N					
C_4	1.59	5.35	3.25	4.2	0.89	0.20	Heated 1 hr. at 60°.
	1.59	5.35	3.25	4.3	0.84	0.20	
	2.00	5.40	3.2	4.3	0.89	0.16	
	2.00	5.40	3.2	4.3	0.90	0.22	
	1.85	8.88	2.85	2.85*	0.45*	0.22	
	1.85	8.88	2.85	2.85*	0.57*	0.18	
A_2	2.09	5.60	3.3	4.4	0.98	0.33	
	2.09	5.60	3.3	4.5	0.85	0.27	
Average				4.35	0.89	0.22	

* Value not used in obtaining average.

ments have been made of the electrophoretic mobility on the acid side of the isoelectric point in buffered solutions (9) and Adolf (10) has measured the mobility of solutions of serum globulin in acids using the U-tube method. The globulin was found to have a mobility of about 50 mhos which was independent of the acid employed.

Measurements were made with the strong acids, hydrochloric, hydrobromic, and nitric. The results are given in Tables V to VII. In the solutions of proteins in the alkali hydroxides employed in transference experiments, the OH ion concentration was never sufficiently great to be a factor in the conduction of the

current. With fibrin in acid solutions, the pH was between 4.0 and 3.0. This is a H ion concentration that is sufficient to be noticeable in the carrying of the current. To evaluate how much of a factor it would be, some rough calculations were made, 350 mhos being used as the mobility of the H ion and the mobility of positively charged fibrin being taken as that of chloride ion. These calculations indicated that with a very few exceptions the H ion carried less than 10 per cent of the current. Since the errors inherent in the experimental results on fibrin solutions are greater than for casein and amount to about 10 per cent, the effect of the H ion was neglected.

TABLE X.

Transport Numbers of Fibrin Solutions in Lactic Acid.

Temperature 25°.

Fibrin preparation.	Fibrin.	A	pH	K	T fibrin⁺.	T lactate⁻.	Remarks.
	per cent	*cc. 0.1 N*					
C_4	1.42	4.65	3.55	6.1	0.89	0.24	Heated 1 hr. at 60°.
	1.42	4.65	3.55	6.0	1.00	0.23	
	1.93	4.65	3.5	6.4	1.00	0.38	Heated 1 hr. at 60°.
	1.93	4.65	3.5	6.2	1.06	0.26	
A_2	1.37	12.67		6.7	1.47	0.25	Not heated.
	1.37	12.67		6.5	1.51	0.28	
	1.96	6.95		4.6*	1.11	0.29	Not heated.
	1.96	6.95		4.4*	1.26	0.28	
Average				6.3	1.29	0.28	

* Value not used in obtaining average.

With the strong acids the transference results are analogous to those obtained with the alkali hydroxides. The sum of the fibrin and anion transport numbers is sufficiently close to 1 to show that these are the only important carriers of the current in the solution. Also the transport numbers of the fibrin vary inversely as the mobility of the anion, as is shown in Table XI by the calculated mobility of the positively charged fibrin being essentially the same in all of the strong acids. This further indicates that the migration of the ionic constituents takes place independently and that

the solutions are completely ionized for the same reasons that have already been given for alkali fibrin solutions.

The calculated mobility of the positively charged fibrin (average 78 mhos) is seen to be about the same as that of potassium ion and very much higher than that of negatively charged fibrin. This difference may perhaps be ascribed to a lower hydration of the positively charged fibrin. However, this is no more than a guess and for the present the cause of the difference is unknown.

Complete Transference Experiments.—For a transference experiment to be completely valid the change in concentration of the

TABLE XI.

Mobility of Fibrin in Alkali and Acid Solutions Calculated from Transference Data.

Temperature 25°.

Cation used	T fibrin$^-$.	T cation.	Λ^0 cation.	Λ^0 fibrin$^-$.
Sodium	0.470	0.50	51.1	45.3
Potassium	0.375	0.58	74.8	44.7
Lithium	0.515	0.44	39.7	42.3
Average				44.0
Anion used	**T fibrin$^+$.**	**T anion.**	**Λ^0 anion.**	**Λ^0 fibrin$^+$.**
Chloride	0.51	0.50	75.8	78.7
Bromide	0.50	0.45	77.8	77.8
Nitrate	0.55	0.36	70.6	86.0*
Average				78.0

* Value not used in obtaining average.

cathode and anode portions should be equal in amount but opposite in sign and the concentration of the middle portion should remain unaltered. With the alkali protein experiments it was not possible to make a complete analysis of all portions because of the use of a lead, lead peroxide electrode as the cathode. Fibrin solutions in the halide acids offered the opportunity to obtain complete transference experiments. The anode consisted of a silver wire and as the current passed through the cell, a film of silver halide deposited on the wire. Thus, no other ionic products than were originally in the solution took part in the carrying of

the current in the anode compartment. Also, since the silver halide adhered to the wire, it was no factor in the analysis of the solution. With hydrochloric acid, small amounts of the silver chloride did come off from the electrode and the transport numbers for the fibrin from the anode analyses are a little low as a result. In Tables V and VI, the results obtained from the analysis of cathode and anode compartments are given for hydrogen chloride and bromide solutions in the designated columns. The agreement for the fibrin transport numbers is quite good, more especially in fibrin hydrobromic acid solutions. The results for the transport numbers of the halide ions are not in such good agreement. Since the concentration changes of halide ions were small, and due to the isoelectric zone, the titration method was not particularly sensitive, the chance for error here is very much greater. The results of the complete transference analyses are a further confirmation that the interpretation of the protein transference data given is correct.

Fibrin in Solutions of Weak Acids.—With the weak acids, formic, phosphoric, and lactic, the results obtained are peculiar. The values for the transport numbers of the fibrin calculated in the usual way are far higher than would be expected from the mobilities of fibrin and the anions of these acids if independent migration were taking place. Indeed, with formic and lactic acids, the fibrin transference is apparently greater than 1. A similar series of results with casein and the alkali earth hydroxides (3) was explained by the formation of negatively charged complex ions between alkali earth metals and casein. Employing a similar explanation for fibrin and the weak acids, we can write as a representative equation:

$$\text{Fibrin } Am = [\text{fibrin } A\ (m - x)]^{x+} + XA^{-} \qquad (3)$$

in which A stands for the anion of a weak acid. This explanation is equivalent to the statement in colloid chemical terminology that there is an adsorption of both undissociated acid molecules and H ions by the fibrin. The findings of Adolf (10) on blood globulins are confirmatory of the hypothesis of complex formation. This author, from a physical chemical study of globulin solutions, concluded that there is complex formation with weak acids. However, the transport numbers of the anions in these solutions,

as calculated from the titration of the solutions, offer a difficulty. The results obtained have the wrong sign, since the sum of the fibrin and anion transport numbers, instead of being unity, adds up to about 1.5 for formate and lactate solutions. Just how this anomaly is to be explained, it is not possible, at the present time, to state.

SUMMARY.

1. Measurements have been made of the electrical transference of solutions of fibrin in dilute alkali and acid. In the solutions of the alkali hydroxides and strong acids used, the results indicate that there is complete ionization of the alkali and acid fibrinates in the sense of the interionic attraction theory of electrolytes.

2. The mobility of the negatively charged fibrin ion or micelle, was calculated to be 44 mhos and that of the positively charged 78 mhos.

3. The electrochemical equivalent of fibrin was found to vary with the method of preparation.

4. In solutions of the weak acids, formic, phosphoric, and lactic, the results indicate the formation of positively charged complex ions of fibrin and the above acids.

BIBLIOGRAPHY.

1. McBain, J. W., and Bowden, R. C., *J. Chem. Soc.*, 1923, cxxiii, 2417. Laing, M. E., *J. Physic. Chem.*, 1924, xxviii, 673. Wintgen, R., *Z. physik. Chem.*, 1922, ciii, 238. Wintgen, R., and Lowenthal, H., *Z. physik. Chem.*, 1924, cix, 382.
2. Greenberg, D. M., and Schmidt, C. L. A., *J. Gen. Physiol.*, 1924, vii, 287.
3. Greenberg, D. M., and Schmidt, C. L. A., *J. Gen. Physiol.*, 1926, viii, 271.
4. Bosworth, A. W., *J. Biol. Chem.*, 1915, xx, 90.
5. Greenberg, D. M., *Univ. Calif. Pub. Physiol.*, 1927, vii, 9.
6. Ferguson, A. L., and France, W. G., *J. Am. Chem. Soc.*, 1921, xliii, 2161.
7. France, W. G., and Moran, W. H., *J. Am. Chem. Soc.*, 1924, xlvi, 19.
8. Scatchard, G., *J. Am. Chem. Soc.*, 1924, xlvi, 2353.
9. Svedberg, I., and Tiselius, A., *J. Am. Chem. Soc.*, 1926, xlviii, 2272. Michaelis, L., and Airila, L., *Biochem. Z.*, 1921, cxviii, 144. Abramson, H. A., *J. Am. Chem. Soc.*, 1928, l, 390.
10. Adolf, M., *Kolloidchem. Beihefte*, 1923, xviii, 223.

MEASUREMENTS OF THE PH OF NORMAL, FETAL, AND NEOPLASTIC TISSUES BY MEANS OF THE GLASS ELECTRODE.*

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INTRODUCTION.

From the point of view of determining quantitatively the pH of freshly excised tissues colorimetric methods cannot be said to have any real value. The methods of vital staining (*cf.* Harde and Henri, 1927) yield different values according to the type of indicator employed.

On general grounds, electrometric methods are preferable but herein difficulties are encountered by reason of the disturbing influence of the metal electrodes commonly employed.

In the present investigation, these difficulties have been overcome and the problem has been approached by employing a glass electrode, the principle of which was first described by Haber (1909), and a technique suitable for the examination of animal tissues worked out more recently by Mrs. Kerridge (1925, 1926).

The employment of a glass electrode in conjunction with the use of liquid air for freezing the tissues immediately after excision, as recommended by Mrs. Kerridge, has in the present investigation been attended with some success.

Details regarding the construction of the glass electrode, the method of using the Lindemann electrometer, and the necessity for complete insulation and protection from stray electric fields will be found in the papers by Mrs. Kerridge to which reference has already been made.

* Communicated by Prof. W. C. M. Lewis. This investigation was undertaken on behalf of the Liverpool Medical Research Organization: Director, Prof. W. Blair Bell, of the University of Liverpool.

The glass electrode is essentially a glass vessel containing two compartments separated by a wall or membrane of thin glass. If this membrane separates two different solutions having a pH given by pH_1 and pH_2 respectively, a potential difference E is developed across the membrane according to the following equation:

$$E = \frac{RT}{F}(pH_1 - pH_2) + E_g \tag{1}$$

where E_g is a potential due to the state of strain of the glass. The following procedure allows us to eliminate E_g and thus to calculate the pH.

The two compartments are filled with different solutions, one of which has a known pH which we shall refer to as pH_0. If the pH of the other solution is represented by pH_1, then the observed E. M. F. of this cell, represented by E_1 will be given by the following expression:

$$E_1 = \frac{RT}{F}(pH_0 - pH_1) + E_g \tag{2}$$

The standard solution characterized by pH_0 is now replaced by the solution or material under investigation, the pH of which will be represented by pH_x. The other solution is still retained in the adjoining compartment.

If the observed E. M. F. of this cell is E_2, we can write

$$E_2 = \frac{RT}{F}(pH_x - pH_1) + E_g \tag{3}$$

On subtracting equations (2) and (3) we obtain finally

$$E_1 - E_2 = \frac{RT}{F}(pH_0 - pH_x) \tag{4}$$

which allows us to calculate the unknown pH_x.

For making the pH measurements the cell employed may be represented thus:

Saturated calomel electrode	Glass electrode	Saturated calomel electrode

The saturated calomel electrodes serve the purpose of making a convenient electrical contact with the two solutions in the glass

electrode and, apart from the possibility of error due to liquid-liquid potentials, do not affect equation (4).

It may be mentioned that a slight modification has been introduced in the type of calomel electrode vessel used in this investigation. In the present instance, the side tubes of the calomel electrode vessels are connected with fine capillary tubes which form the connecting link between the calomel vessels and the solutions in the glass electrode. By working in this manner, diffusion of calomel solution into the glass electrode is reduced to a negligible amount, and measurements can thus be made with confidence on very small quantities of material.

1. pH Determinations of Normal and Neoplastic Tissues.

By use of the technique described, pH measurements have been made of malignant tumors of a number of American and English

TABLE I.

pH of Six American and Twelve English Rat Tumors (Flexner) at 18°.

	pH						Mean pH value.
American rats...	6.75	6.85	6.88	6.74	6.74	6.86	6.80
English rats.....	6.82 6.83	6.78 6.85	6.64 6.78	6.82 6.84	6.78 6.76	6.86 6.80	6.79

white rats, together with similar measurements of human innocent and malignant neoplasms. In all cases, the tissues were thrown into liquid air immediately after excision.

The time interval between the removal of the tissue from the liquid air and the commencement of a pH measurement was usually about 2 minutes, after which time a steady reading was obtained. Small slices of tissue weighing no more than about 0.25 gm. were employed for the measurements and were placed in the glass electrode, immediately after removal from the liquid air, without having been ground or minced.

As we have already seen, the pH determinations are made possible by the use of a standard solution of known pH. In the work herein recorded, this standard solution contained 0.05 M potassium hydrogen phthalate. The pH of this solution at the

temperature employed throughout this investigation, namely 18°, has been taken to be 3.97. This value has been checked from time to time by measurements with a quinhydrone electrode.

The results obtained in the pH measurements of rat tumors are given in Table I, in which the figures refer to individual rats. The English rats were obtained by purchase from the Glaxo Company, while the American rats were kindly supplied by Dr. Carter Wood.

In all these cases, the tumors were derived from inoculation beneath the skin.

TABLE II.

pH of Human Innocent Neoplasms at 18°.

	pH	
	Neoplasm.	Surrounding tissue.
Inflammatory tissue from groin	6.87	7.14
Benign muscle tumor, fibromyoma	6.90	7.02
Myofibroma of ovary	6.83	7.07 (Normal ovary.)
Lymphoid tissue	6.87	
Ovarian cyst	6.95	
" "	7.04	
Uterine fibroid	6.94	7.09
" polyps	6.84	7.09
Parenchymatous goiter	7.00	7.16
Lipoma of arm	6.95	7.15
Mean	6.92	7.10

Slices of tissue were removed from the necrosed and non-necrotic portions of the tumor in each case, but no difference was detected as regards the pH.

From the results in Table I, it will be seen that the average value of the Flexner rat carcinoma is 6.80. As a control, measurements were made on normal organs; *e.g.*, liver and spleen in the case of the above rats, the average pH of which was found to be 7.10. Experiments have been carried out to determine the pH of human innocent and malignant neoplasms, and in several cases it has been possible to effect a comparison of the reaction of the neoplasm and that of the surrounding normal tissue.

It is of interest to note that the surrounding tissue in every case is less acid than the neoplastic tissue. Furthermore, it will be seen in Table II that innocent neoplasms have an average pH of 6.92, while the mean value in the case of the malignant neoplasms is 6.78. The latter value agrees well with the average pH of the malignant rat tumors.

The results obtained for the pH of human innocent neoplasms are recorded in Table II, and the corresponding data for human malignant neoplasms in Table III.

The pathological examinations were carried out in the Department of Pathology of the University of Liverpool.

TABLE III.

pH of Human Malignant Neoplasms at 18°.

	pH	
	Neoplasm.	Surrounding tissue.
Epithelioma of skin of breast	6.82	7.13
" " lip	6.72	7.00
Carcinoma of breast	6.72	7.00
Sarcoma of groin	6.86	
Cancer " "	6.74	
Chronic mastitis of breast	6.85	7.10
Carcinoma of omentum with secondary carcinoma of pelvis. (a) Omentum	6.82	
Carcinoma of omentum with secondary carcinoma of pelvis. (b) Pelvis	6.85	
Mean	6.78	7.04

2. pH Measurements of Fetal Somatic Tissues.

In order to compare the foregoing results on the reaction of innocent and malignant neoplasms with rapidly growing embryonic tissues, pH measurements have been made on fetal blood and fetal somatic tissues in the case of rabbits at various stages of pregnancy, and these are compared with the maternal tissues.

The samples of blood and amniotic fluid were always withdrawn and retained under liquid paraffin in order to prevent loss of carbon dioxide, while the various tissues were treated with liquid air as already mentioned.

These pH determinations, which are summarized in Table IV, clearly show that fetal somatic tissues possess a lower pH than the corresponding maternal tissues and that, in general, the fetal tissues become progressively less acid with increasing age of the fetus.

It is interesting to note that a rapid change towards the normal state as represented by the fully grown rabbit, is observed with

TABLE IV.

pH Measurements of Normal and Fetal Somatic Tissues of Seven Pregnant Rabbits at 18°.

No. of days pregnant	17	20	23	24	26	26	29
Maternal heart	7.36						7.40
Fetal heart		5.78	6.82	7.06			7.06
Maternal liver	7.21				7.06	7.15	7.19
Fetal liver		5.98	6.58	7.05	7.01	6.98	7.03
Maternal kidney	7.14					7.05	7.14
Fetal kidney		5.55	6.68	6.78	6.35	6.67	6.43
Placenta		6.12	6.59		6.76	6.87	6.97
Amniotic fluid	5.99	5.74					
Maternal blood.							
Venous	7.20	7.18				7.15	
Arterial		7.22		7.35		7.21	
Fetal heart blood				6.50		6.88	

newly born rabbits. For example, the following data indicate that in less than *12 hours* after birth, the blood and tissues of the young rabbit have the same reaction as exhibited by a fully grown normal rabbit.

Age.	Liver.	Kidney.	Heart.	Blood.
Newly born				7.19
12 hrs	7.11	7.16	7.41	7.35

In a similar manner, the pH of the blood of some young guinea pigs has been measured and is recorded below. In this case, although the pH of the newly born is somewhat low, there is a very rapid change towards the normal state.

Age.	pH of blood.
1 hr.	6.02
3 hrs.	6.40
7½ "	7.16

It should be mentioned that Mlle. Mendeléef (1923) observed that the fetal blood and amniotic fluid of a guinea pig at half term was distinctly more acid than the maternal blood, which observation is supported by the results of the present investigation. However, Mlle. Mendeléef states that a period of 6 days from birth elapses before the blood of a guinea pig possesses a normal reaction, whereas the results given above would indicate that this period is probably less than half a day.

DISCUSSION.

The foregoing results show that both innocent and malignant neoplasms have a distinct acid reaction, the average pH of innocent neoplasms being 6.92, while that of malignant neoplasms is 6.80.

In addition, it appears that the normal tissue surrounding the malignant growths has a somewhat lower pH (7.04) than that surrounding the innocent neoplasms, which is usually about 7.10. Qualitatively the above results might be expected owing to the effect of glycolysis in producing excessive amounts of lactic acid. This effect would be most marked in the case of malignant tumors, and consequently a diffusion of lactic acid into the surrounding tissue may cause the latter to become more acid.

It is instructive to compare the pH data for neoplasms with those obtained for fetal somatic tissues which are also characterized by a distinct acid reaction. Such a comparison would suggest that an acid reaction goes hand in hand with rapid growth of tissues.

In addition to the work of Mlle. Mendeléef (1923) on rabbit fetal tissues, mention should be made of an investigation by Harde and Henri (1927) who state that the grafted sarcomas of rats, the spontaneous carcinomas of mice, and the embryos of mice show a distinct acid reaction. Qualitatively, this is in accord with the results presented in this paper, but Harde and

Henri do not claim any accuracy for their determinations (which give only relative results) since they were obtained by the method of vital staining.

In conclusion, it has been shown that the necrosed and non-necrotic portions of tumors possess the same pH. The writer, therefore, is unable to confirm Woglom's (1924) finding that the necrotic portion is distinctly alkaline.

SUMMARY.

1. By means of the glass electrode in conjunction with the use of liquid air for freezing tissues, pH determinations have been made of normal, fetal, and neoplastic tissues at 18°.

2. In the case of Flexner rat carcinoma, the average pH is 6.80, while the average pH of normal organs, *e.g.* liver and spleen, is 7.10.

3. The average pH of human innocent neoplasms is 6.92, while that of human malignant neoplasms is 6.78. These values are lower than the pH of the surrounding normal tissue.

4. No difference has been detected in the pH of the necrosed and non-necrotic portions of tumors.

5. Rabbit fetal tissues have an acid reaction which varies with the age of the fetus; *i.e.*, the pH *increases* with increasing age of the fetus, and in the case of both rabbits and guinea pigs, the reaction becomes normal within about 12 hours of birth.

The writer wishes to express his indebtedness to Mrs. Kerridge for valuable information regarding certain points in the preparation of the glass electrodes.

BIBLIOGRAPHY.

Haber, F., and Klemenziewicz, Z., *Z. physik. Chem.*, 1909, lxvii, 385.
Harde, E., and Henri, P., *Compt. rend. Soc. biol.*, 1927, xcvi, 535.
Kerridge, P. T., *Biochem. J.*, 1925, xix, 611.
Kerridge, P. T., *J. Sc. Instruments*, 1926, iii, 404.
Mendeléef, P., *Compt. rend. Soc. biol.*, 1923, lxxxviii, 146, 291.
Woglom, W. H., *J. Cancer Research*, 1924, viii, 34.

STUDIES ON GLUTELINS.

IV. THE GLUTELINS OF CORN (ZEA MAYS).

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Osborne in 1897 (1) published the results of analyses of the various proteins in the yellow corn kernel. In this paper the presence of a glutelin in corn was demonstrated for the first time. The quantity found was equal to 3.15 per cent of the corn-meal. Later Osborne and Clapp (2) made a more complete study of the composition of this glutelin. They were not certain, however, whether this glutelin preparation was a single protein or a mixture. Showalter and Carr (3) observed that corn with a high protein content contains more zein and less glutelin than corn with a low protein content. Their glutelin figures, however, are calculated by difference, and therefore may not be strictly conclusive.

By applying our method with modifications to the preparation of corn glutelin, we have been able to detect the presence of two glutelins, an α- and a β-glutelin, as we had previously found in the wheat kernel (4). On account of the small quantity of β-glutelin present, analyses were made on the α-glutelin only. The α-glutelin separates from 0.2 per cent sodium hydroxide at 3 per cent of saturation with ammonium sulfate; the β-glutelin separates at 16 per cent of saturation.

The presence of relatively large quantities of starch in cereals usually presents considerable difficulties in the preparation of glutelins. Wheat flour is the only one from which most of the starch can be easily washed out by first making a dough. The gluten remaining contains practically all the glutelins. In other cereals the application of the alcohol-alkali extraction method of Fleurent (5) is very useful, because the bulk of the starch, which

makes filtrations slow and cumbersome, is eliminated at the start. The percentage of alcohol and alkali in the alcohol-alkali mixture should be adjusted so that the prolamin of the cereal under investigation readily dissolves, and still the glutelin stays in solution; 50 to 70 per cent alcohol by volume will serve well in most cases.

Preparation of Corn Glutelins.

In our first attempt to prepare corn glutelin, corn-meal was extracted with 10 per cent sodium chloride solution until no more nitrogenous material was being removed. The extraction was then continued with 85 per cent alcohol.

It was found, however, that cold alcohol did not extract all the zein. The use of heat was avoided on account of the possible loss resulting from partial denaturation of the proteins. As another source of corn glutelin, "corn gluten," a dry, commercial product, was used, but after the zein was removed by extraction with alcohol at 55–60°, the residue yielded no glutelin when extracted with alkali. Not much better results were obtained when a moist corn gluten product was similarly extracted. A glutelin preparation was finally obtained from corn-meal by using a combination of Fleurent's method and our ammonium sulfate method (4). The meal was first extracted with 60 per cent alcohol containing 0.2 per cent sodium hydroxide. The glutelin is precipitable from such an extract by the addition of a small quantity of ammonium sulfate. It is simpler at this stage, however, to add hydrochloric acid until the extract has a pH value of from 6.7 to 6.8. The precipitate which separates on the addition of hydrochloric acid was dissolved in the alcoholic sodium hydroxide solution and reprecipitated by acidification. After three reprecipitations the glutelin was washed with distilled water. If the glutelin does not separate sharply from the aqueous suspension, it is advisable to adjust the pH of the mixture to 6.8 by the addition of a few drops of hydrochloric acid. In all cases the precipitates were separated by centrifugalization.

At this stage the method of precipitating the glutelin from an alkaline solution with ammonium sulfate was applied. The water-washed precipitate was dissolved in 0.2 per cent aqueous sodium hydroxide, and the glutelin was precipitated by adding a saturated

solution of ammonium sulfate until 3 per cent of saturation was reached. A quicker and sharper separation of the protein results when a slight excess of ammonium sulfate is added (4 to 5 per cent

TABLE I.

Distribution of Nitrogen in the α-Glutelin of Corn as Determined by the Van Slyke Method.

Corrected for the solubility of the bases.

	Average of two determinations.
	per cent
Amide N	7.73
Humin N	1.69
Cystine N	2.04
Arginine N	15.11
Histidine N	2.81
Lysine N	7.99
Amino N in filtrate from bases	59.64
Non-amino N in filtrate from bases	4.27

TABLE II.

Amino Acids in the α-Glutelin of Corn.

Calculated on an ash- and moisture-free basis.

	per cent
Cystine	2.81
Arginine	7.56
Histidine	1.67
Lysine	6.71

TABLE III.

Elementary Composition of the α-Glutelin.

Calculated on an ash- and moisture-free basis.

	per cent
C	54.07
H	6.94
S	1.04
N	16.10
Ash	0.217

of saturation). The precipitate was thoroughly washed successively with a 5 per cent saturated solution of ammonium sulfate, distilled water acidified to pH 6.8, and 70 per cent alcohol. It

was finally dried with absolute alcohol and ether in the usual way. The yield was about 0.7 per cent, calculated on the basis of the meal used.

The alkaline supernatant liquid from which the α-glutelin had separated contained a second glutelin which separated on further addition of ammonium sulfate to 16 per cent of saturation. The quantity of this glutelin obtained was small.

The isoelectric point of the α-glutelin, which was determined by the method described in a previous publication from this laboratory (6), was found to be at pH 6.45. In general, the solubility of glutelins in the buffer solutions used in this method is rather low and requires more time than that specified in our work with globulins and albumins. To avoid decomposition of the glutelin, a few drops of toluene were added to the buffer solutions.

The composition and distribution of nitrogen in the α-glutelin are given in Tables I to III.

SUMMARY.

Corn (*Zea mays*) contains two glutelins (α- and β-) which are precipitable from alkaline solution by the addition of ammonium sulfate to 3 per cent and 16 per cent of saturation, respectively.

Analyses of the α-glutelin by the Van Slyke method showed the following percentages: amide N, 7.73; cystine N, 2.04; arginine N, 15.11; histidine N, 2.81; lysine N, 7.99; amino N in filtrate from bases, 59.64.

The isoelectric point of the α-glutelin was found to be at pH 6.45.

BIBLIOGRAPHY.

1. Osborne, T. B., *J. Am. Chem. Soc.*, 1897, xix, 525.
2. Osborne, T. B., and Clapp, S. H., *Am. J. Physiol.*, 1908, xx, 477.
3. Showalter, M. F., and Carr, R. H., *J. Am. Chem. Soc.*, 1922, xliv, 2019.
4. Csonka, F. A., and Jones, D. B., *J. Biol. Chem.*, 1927, lxxiii, 321.
5. Fleurent, E., *Compt. rend. Acad.*, 1896, cxxiii, 327, 755.
6. Csonka, F. A., Murphy, J. C., and Jones, D. B., *J. Am. Chem. Soc.*, 1926, xlviii, 763.

THE RELATIVE STABILITY OF VITAMIN A FROM PLANT SOURCES.*

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Osborne and Mendel,[1] Hopkins,[2] Drummond and Coward,[3] and Zilva[4] have reported experiments upon the stability or destruction of vitamin A as it exists in butter fat or in cod liver oil. Less work has been recorded upon the stability of the vitamin A of plants although some observations by Steenbock and Boutwell[5] and by Osborne and Mendel[6] have seemed to indicate that vitamin A exists in plant tissues in a more stable form than in animal fats.

The present investigation has included experiments, first to determine the effects of heating, in the presence or absence of oxygen and at different hydrogen ion activities, upon the vitamin A occurring in a primary or plant source, and then parallel experiments to compare the stability of this vitamin as obtained from a plant and an animal source.

General Procedure Employed in These Experiments for Measuring Relative Amounts of Vitamin A.—The quantitative method for determining vitamin A in these experiments was essentially that described by Sherman and Munsell.[7] Albino rats were placed on a vitamin A-free diet at 28 days of age and the feeding of this diet continued until the surplus vitamin A stored in the animal's

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[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 379.

[2] Hopkins, F. G., *Biochem. J.*, 1920, xiv, 725.

[3] Drummond, J. C., and Coward, K. H., *Biochem. J.*, 1920, xiv, 734.

[4] Zilva, S. S., *Biochem. J.*, 1924, xviii, 881.

[5] Steenbock, H., and Boutwell, P. W., *J. Biol. Chem.*, 1920, xli, 163.

[6] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 549.

[7] Sherman, H. C., and Munsell, H. E., *J. Am. Chem. Soc.*, 1925, xlvii, 1639.

body was depleted, as evidenced by cessation of growth and by the incipient appearance of other symptoms characteristic of vitamin A deficiency. Graded portions of the test foods whose relative vitamin A values were to be compared were then fed as supplements to the vitamin A-free diet. All of the feeding experiments were made in pairs upon litter mates of essentially the same weight, one being fed one material, the other the same material but differently treated or a different material similarly treated, as the case might be. Those portions of the two samples which, fed daily during an 8 week experimental period, gave the same small but definite gain in weight, *i.e.* those portions which contained the same amount of vitamin A, were thus determined; and from these values the amount of destruction of the vitamin due to any particular treatment was calculated.

The basal vitamin A-free diet fed in all experiments consisted of: extracted casein 18, corn-starch 67, yeast 10, Osborne and Mendel salt mixture[8] 4, and sodium chloride 1 per cent. Comparisons made between animals which did and others which did not receive direct irradiation with ultra-violet light or irradiated cholesterol with their basal diet both in the course of these experiments and in connection with other work,[9] showed that under the conditions of these experiments vitamin D was not a limiting factor in the vitamin A determinations.

Each animal was kept in a separate cage with raised screen bottom during the experimental period. The basal diet was fed *ad libitum* and a careful record kept of the food consumed. The test food was fed separately, the prescribed "daily" allowance being fed regularly each day except Sundays, and its complete consumption was insured.

I. A Study of the Stability of Vitamin A in a Plant Material When Heated for 4 Hours at 100° under Anaerobic or Aerobic Conditions.

Tomato juice was selected as the primary source of the vitamin in this series of experiments. The juice was obtained by filtering canned tomatoes through cheese-cloth to separate the greater part of the solid material and this filtrate then passed through filter paper. A clear yellow liquid was thus obtained.

[8] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.
[9] Sherman, H. C., and Hessler, M. C., *J. Biol. Chem.*, 1927, lxxiii, 113.

For the study of the stability of the vitamin in the absence of oxygen the following procedure was finally adopted. The juice was placed in a 1 liter flask provided with a rubber stopper through which passed a reflux condenser with its open end protected from the air by a water seal. Nitrogen freed from oxygen by passing through alkaline pyrogallol and then over 12 inches of red-hot copper gauze, was bubbled through the liquid for 2 hours. At the end of this 2 hour period half of the juice was withdrawn through a properly arranged delivery tube to an evacuated bottle and the remainder heated on a steam bath for 4 hours. The nitrogen was permitted to bubble through the liquid during the heating process at the rate of about one bubble per second. A thermometer within the flask showed that the temperature was maintained at 97° ± 2°. At the end of 4 hours the flask was cooled with running water and the tomato juice then removed to an evacuated bottle. Both the unheated and heated samples were stored in a refrigerator except when in use. When portions were removed for the feeding tests the amount withdrawn was displaced by nitrogen.

To prevent the entrance of air through any of the rubber connections or through the stopper of the flask during the nitrogen treatment, all rubber connections were shellacked and the stopper of the flask was sealed with sealing-wax. That anaerobic conditions were obtained during the heating of the samples under these conditions was indicated by tests carried out with distilled water in the flask. After bubbling the purified nitrogen through the apparatus for varying lengths of time it was found that the distilled water gave negative tests for oxygen with reduced indigo after 55 minutes passage of the nitrogen gas. Although by these tests the removal of all free and dissolved atmospheric oxygen from the apparatus and the distilled water was insured within 1 hour's time, the passage of the oxygen-free nitrogen during each treatment of the tomato juice was continued for 2 hours before the unheated portion was removed and heat was applied to the remainder of the sample.

With the samples obtained by the method finally adopted where the rigorous precautions described above were taken for excluding atmospheric oxygen, it was found that 7.0 cc. of heated juice daily were required to produce the same gain in weight as 5.8 cc. of unheated juice daily. Thus, with the greatest care taken to exclude

oxygen from the system in which the vitamin was heated, about 17 per cent of the vitamin A was destroyed in 4 hours heating at 97° ± 2°, at the natural acidity of the tomato juice; *viz.*, about pH 4.2.

To determine the effect of heating the vitamin in the presence of oxygen, experiments were undertaken in which the tomato juice was heated on a steam bath under a reflux condenser for 4 hours at 97° ± 2° with air aspirated through the sample at the rate of about one bubble per second throughout the heating. The animals receiving 7.0 cc. of this heated juice daily made approximately the same growth as the animals receiving the 5.8 cc. of the unheated juice daily. No measurable difference in destruction of vitamin A was thus found between the experiments in which the vitamin was heated in the absence of oxygen and those in which oxygen was present abundantly throughout the period of heating.

II. Effect of Change of pH on the Stability of Vitamin A in a Plant Material When Heated under Anaerobic Conditions for 4 Hours at 100°.

It has been found that vitamin B in tomato juice is more rapidly destroyed if the acidity of the medium is decreased or if the juice is made faintly alkaline.[10]

It was desired to determine by quantitative experiments whether a similar change of hydrogen ion activity would increase the destruction of vitamin A as it exists in tomato juice.

In these experiments the canned tomatoes were pulped and filtered through two thicknesses of cheese-cloth, thus yielding a juice somewhat turbid but richer in vitamin A.

The portion to be subjected to the heat treatment under changed hydrogen ion activity was brought to the desired pH by adding approximately N NaOH solution until the E.M.F. measured on a Leeds and Northrup potentiometer corresponded to a pH of 9.2. The juice thus prepared was heated in parallel with juice of natural acidity (approximately pH 4.2). The two samples were heated on a steam bath for 4 hours at 98° ± 2° under an atmosphere of nitrogen as already described. The juice in the two flasks was then quickly cooled and, to the sample whose pH had been changed, hydrochloric acid was added in amount just sufficient to neutralize

[10] Sherman, H. C., and Burton, G. W., *J. Biol. Chem.*, 1926, lxx, 639.

the sodium hydroxide that had previously been introduced. 2 cc. daily of the juice heated at pH 4.2 were fed to rats and a comparison of the growth resulting made with the growth of litter mates receiving exactly the same amount (correction being made for increase in volume due to acid and alkali added in adjusting the pH) of the juice heated at pH 9.2. The total average weight gain for the 8 week period was 47 gm. for the juice which had been heated at pH 4.2 and 45 gm. for the juice which had been heated at pH 9.2. Thus the change of hydrogen ion activity from pH 4.2 to pH 9.2 did not appreciably influence the rate of destruction of vitamin A.

III. Comparison of Effect of Heat on Vitamin A from a Plant and an Animal Source.

Since results of the first series of experiments had shown vitamin A in a plant material to be relatively stable, it seemed desirable to compare the stability of the vitamin from animal and plant sources when heated under conditions as nearly identical as possible, including the use of the same solvent. It was found that an extract fairly rich in vitamin A was prepared by shaking dry powdered spinach leaves with olive oil. Since a solution of butter fat in olive oil could readily be obtained, these two olive oil preparations furnished suitable materials for the present study.

The dried powdered spinach was shaken mechanically for 8 hours with twice its weight of virgin olive oil, then allowed to settle, and the oil filtered through several thicknesses of fine filter paper. Clear butter fat was prepared from butter in the usual way and dissolved in an equal weight of the same olive oil.

These olive oil solutions containing the vitamin A of spinach leaves and of butter fat, respectively, were heated for 4 hours at $97° \pm 2°$ under anaerobic conditions, essentially as described earlier in this paper. As an extra precaution to insure removal of all dissolved oxygen from the oil solutions the time of preliminary bubbling of nitrogen was increased from 2 hours to 3 hours.

Frequently repeated quantitative determinations of the vitamin A values by the feeding method already described, showed that of the vitamin A of the spinach about 20 per cent, and of that of the butter about 33 per cent, were destroyed during the 4 hours heating.

The quantitative feeding experiments were so numerous and

their results so consistent as to establish these measurements of the percentage destruction of the vitamin with considerable accuracy and thus to show definitely a greater stability of the vitamin A in the olive oil extract of spinach than in the olive oil solution of butter fat under the conditions of these experiments.

Until more is known regarding the possible presence and relative activity of oxidizing substances in olive oil, spinach, and butter fat, any discussion of the question as to whether the destruction of vitamin A here observed may have been due to an oxidation reaction, even though dissolved and atmospheric oxygen had been excluded, would, in our judgment, be premature.

SUMMARY.

Vitamin A from plant sources is shown to be relatively stable. When tomato juice was heated at 97° ± 2° for 4 hours in an atmosphere of nitrogen, the destruction of vitamin A was found to be about 17 per cent; the destruction was not measurably increased when air was bubbled through the juice continuously during the heating process.

The stability of vitamin A in tomato juice, when heated at 98° ± 2° for 4 hours in an atmosphere of nitrogen, was found to be the same at pH 9.2 as at pH 4.2, the normal acidity of the juice.

When heated under anaerobic conditions for 4 hours at 97° ± 2°, the vitamin A contained in an olive oil extract of dry spinach was found to be somewhat more stable than was the vitamin A in an olive oil solution of butter fat heated under the same conditions. The destruction of the vitamin A from spinach was about 20 per cent; of that from butter, about 33 per cent.

ON THE ALLEGED CONVERSION OF FAT TO CARBOHYDRATE.

I. THE METABOLISM OF ACETIC ACID.*

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INTRODUCTION.

No convincing experimental evidence that the mammal is able to convert the fatty acid portion of the fat molecule into carbohydrate has been put forward. In spite of this, a large school of physiologists in this country and abroad has accepted such a physiological transformation as a proved fact.

There is general agreement that glycerol acts as a glucogenetic agent. Cremer (1) and Lüthje (2) were able to recover 40 per cent of the glycerol ingested by diabetic animals as extra sugar in the urine while Chambers and Deuel (3) observed a practically complete conversion of this alcohol to glucose in two phlorhizinized dogs. On the other hand, the bulk of experimental evidence indicates that the fatty acid gives rise to β-hydroxybutyric acid and not to sugar. Lusk (4) has found no increase in the D:N ratio of phlorhizinized dogs in which the fat metabolism has been largely increased either by mechanical work or by shivering. Not only was there no excretion of urinary sugar which might be traced to a synthesis from fatty acids but even that which one would expect from the glycerol metabolized did not appear in the urine. Lusk[1] has suggested that when glycerol is metabolized with the fatty acid chain in phlorhizinized animals, it is oxidized completely to CO_2 and H_2O.

* A preliminary report of this work was published in *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 667.

[1] Personal communication from Professor Lusk.

If the fatty acid having an even numbered carbon chain were capable of conversion into glucose, it is probable that its oxidation products would be the intermediates in this transformation. As a result of β-oxidation of the fatty acid, it is generally believed that acetic acid may be produced which ultimately would be oxidized to CO_2 and H_2O. If fat is a potential glucose former, then acetic acid should serve as a source of glucose. Ringer and Lusk (5) have reported one experiment in which 10 gm. of sodium acetate (equivalent to 4.41 gm. of glucose) were given to a phlorhizinized dog without any change in the D:N ratio or any increase in the glucose excretion being observed. On the contrary, Geelmuyden (6) has reported several experiments on phlorhizinized dogs which he believes are proof of the formation of glucose from this acid. His results are not convincing and do not offer strong support for the contention that such a synthesis may occur. His experimental animals received a constant carbohydrate-containing diet (resulting in the basal D:N ratio of from 6.0 to 7.0 or higher) and the increased sugar excretion was not greater after the administration of sodium acetate than on some of the control days. In one experiment on which his conclusions are based, the total urinary glucose alone is determined without any regard being taken of the nitrogen excretion or of the D:N ratio. Geelmuyden has suggested that acetic acid is not immediately converted into glucose in some cases but that the increased glucose excretion occurs on the day following its administration. That the conversion into glucose of a substance so rapidly metabolized as sodium acetate should be so retarded is without precedent when compared with other intermediate substances capable of yielding glucose.

Thunberg (7) has postulated that acetic acid is convertible into succinic acid in the following manner in the animal organism.

$$2\ CH_3COOH \rightarrow \begin{array}{l} CH_2COOH \\ | \\ CH_2COOH \end{array} + H_2O.$$

If such a transformation is possible in the mammal, it would be suggestive of the probable intermediate reactions involved in the change from fatty acid to sugar since it has been shown by Ringer, Frankel, and Jonas (8) that succinic acid is a glucose former. However, Clutterbuck (9) using the minced tissues of rabbits in

some recent work has been unable to confirm the results of Thunberg.

Acetic acid has been identified as a normal constituent in horse urine (10) and in human urine (11). Schotten (10) recovered considerable amounts of acetate in the urine after giving 25 gm. doses to dogs but noted a large increase in the Na_2CO_3 content of the urine which would indicate that the larger portion of the ingested sodium acetate was oxidized to CO_2. Dakin (12) has obtained similar results and has shown that acetic acid is not changed to oxalic acid in the course of its transformation to carbonic acid.

In view of the contradictory evidence as to the fate of acetic acid and because of its importance as a possible intermediate product of fat oxidation, the following experiments were carried out to throw further light on its metabolism.

General Procedure.

Fasting female dogs were used in the experiments reported here. Phlorhizin was administered by the technique usually followed in this laboratory (13). The urine was collected from the cages and by catheter in 24 hour periods until the animal was completely diabetic, after which time 8 to 12 hour samples were taken before and after the administration of CH_3COONa or glucose.

Since it was found in preliminary experiments that the oral administration of sodium acetate often produces a violent catharsis which may prevent the quantitative absorption of the salt, the acetate was injected subcutaneously or intraperitoneally in amounts from 14 to 27.4 gm. dissolved in 80 to 140 cc. of water. Sterile technique was employed throughout.

To prove that any glucose formed by the metabolism of the injected acetate would be eliminated in the urine and result in an increased D:N ratio, control experiments were carried out on the same animals in which glucose was administered orally or subcutaneously whereby it was demonstrated that such glucose would be excreted approximately quantitatively in the urine.

Total nitrogen was determined by the usual macro-Kjeldahl technique. The Bertrand method was used for the quantitative determination of urinary glucose. In order to ascertain whether any of the injected sodium acetate was excreted by the kidney

unoxidized, the amount of volatile acids in the urine was determined as follows: To 150 cc. of the urine, 20 cc. of concentrated H_2SO_4 were added and this mixture was distilled until 130 cc. of distillate were obtained. After the distillate had been made up to 200 cc., the acid content was determined on an aliquot portion by titration with 0.1 N alkali, phenolphthalein being used as an indicator. By this procedure with known solutions of sodium acetate, 95 to 96 per cent of the added substance was recovered. Quantitative results were obtained when sodium acetate was added to normal or diabetic urine in which the volatile acid had previously been determined on an aliquot portion. Urine of normal fasting animals was found to contain a small amount of volatile acid, while much higher values were obtained from the urine of diabetic animals. The presence of chlorides in sufficient amount will result in the distillation of appreciable quantities of HCl, thus giving an erronous value when the volatile acid is considered wholly as acetic acid. This difficulty was minimized in our experiments since the chloride excretion was reduced to a low figure by fasting. Likewise we were interested in the increase of the volatile acid excretion after the acetic acid administration, the calculation of which would not be influenced by a small constant quantity of NaCl present.

In the following experiments the extra sugar in the urine has been calculated in each case by considering the prevailing D:N ratio for the acetate period as the average of the fore and after periods. The nitrogen of the experimental period multiplied by the prevailing ratio gives the glucose to be expected from the protein metabolism of the time. The glucose actually found in excess of this is calculated as extra sugar.

EXPERIMENTAL.

Cathartic Action of Sodium Acetate.—It is not generally recognized that sodium acetate may act as a violent purge. This fact soon became evident when we attempted to administer this salt by the oral route in the early experiments which are not recorded here. The ingestion of this substance was always followed within a remarkably short time by the elimination of a large amount of fluid from the bowel. In five later experiments with three normal dogs which had been fed on the previous night, the oral introduc-

tion of 27 gm. of sodium acetate in 250 to 300 cc. of H_2O was followed in 11 to 26 minutes (average 19 minutes) by the ejection of a large amount of fluid from the intestines. Vomiting also

TABLE I.

Effect of Administration of Sodium Acetate on Metabolism of a Phlorhizinized Dog.

Dog 175. Weight 12.9 kilos.

Date.	Length of period.	Glucose for period.	Nitrogen. Period.	Nitrogen. Hourly.	D:N	Glucose ingested or glucose equivalent.	Extra glucose.	Volatile acid (0.1 N). Period.	Volatile acid (0.1 N). Hourly.	Remarks.
Mar., 1927	*hrs.*	*gm.*	*gm.*	*gm.*		*gm.*	*gm.*	*cc.*	*cc.*	
3	15.53	30.94	6.370	0.410	4.86			2412	155	Phlorhizin daily from Feb. 28.
3	8.70	15.94	3.580	0.411	4.45			1500	172	
4	15.30	25.76	6.186	0.404	4.16			2400	158	
4	6.00	10.28	2.480	0.413	4.10			1224	204	
4	6.38	12.19	2.612	0.409	4.74			990	155	
5	11.62	21.84	5.250	0.452	4.16	12.01	0	2412	207	27.24 gm. $NaCH_3CO_2$ subcutaneously.
5	6.00	10.51	2.920	0.488	3.60			948	158	
5	6.00	9.73	2.714	0.452	3.59			768	128	
6	12.33	20.58	4.935	0.400	4.17	7.05	2.27	2616	212	16.0 gm. $NaCH_3CO_2$ intraperitoneally.
6	5.67	8.86	2.310	0.407	3.83			822	145	
6	6.00	8.66	2.383	0.397	3.63			660	110	
7	12.22	28.05	3.950	0.323	7.10	14.00	13.99	954	78	14.0 gm. glucose by stomach tube.
7	11.71	13.51	3.870	0.331	3.49			1662	142	
8	12.40	13.48	3.725	0.304	3.62	6.39	0.26	1338	108	14.5 gm. $NaCH_3CO_2$ intraperitoneally.
8	6.31	6.61	1.835	0.291	3.60			720	114	

occurred as a usual thing. In one animal 8.18 gm. of volatile acid were found in the fecal fluid while 12.71 gm. were found in the vomitus. This indicates the extreme rapidity with which the acetate may be carried the entire length of the gastrointestinal

tract. It was obvious from these results that one may not rely on quantitative absorption of the acetate when introduced by the oral route. Gaebler (14) has obtained a similar purgative effect with potassium acetate.

Effect of Injection of Sodium Acetate on Glucose Excretion of Diabetic Dogs.—Sodium acetate was administered subcutaneously and intraperitoneally in amounts varying from 14.5 to 27.4 gm. (equivalent to 6.39 to 12.09 gm. of glucose) in twelve experiments

TABLE II.

Effect of Subcutaneous and Intraperitoneal Administration of Sodium Acetate on Glucose Excretion and D:N Ratio of Phlorhizinized Dogs.

Dog No.	Weight of dog.	D:N ratio. Control period I.	Acetate period.	Control period II.	Prevailing ratio.	Sodium acetate.	Glucose. Equivalent introduced.	Extra glucose.	Remarks.
	kg.					*gm.*	*gm.*	*gm.*	
120	5.9	3.34	2.96	3.18	3.26	15.0	6.62	0	Subcutaneously.
120	5.9	3.18	2.94	2.99	3.08	15.0	6.62	0	"
169	9.2	3.50	3.63	3.81	3.66	27.4	12.09	0	"
169	9.2	3.81	3.77	3.30	3.56	16.0	7.05	0.97	Intraperitoneally.
180	15.2	4.77	5.14	4.28	4.53	27.2	12.00	5.14	Subcutaneously.
180	15.2	4.28	4.28	3.79	4.04	27.2	12.00	2.10	"
180	15.2	3.79	3.89	3.33	3.56	27.2	12.00	2.78	Intraperitoneally.
176	8.4	3.71	3.61	3.41	3.56	15.0	6.62	0.40	Subcutaneously.
176	8.4	3.41	3.84	3.50	3.46	27.2	12.00	2.08	Intraperitoneally.
175	12.9	4.74	4.16	3.60	4.17	27.2	12.00	0	Subcutaneously.
175	12.9	3.59	4.17*	3.83	3.71	16.0	7.05	2.27	Intraperitoneally.
175	12.9	3.49	3.62	3.60	3.54	14.5	6.39	0.26	"

* Later experiment showed no extra glucose.

on five phlorhizinized dogs. A typical experiment is given in Table I which summarizes the experimental data on Dog 175. Table II contains a summary of the other experiments.

When sodium acetate was administered subcutaneously to Dog 175, no extra sugar was excreted in the urine. After the intraperitoneal injection of this salt solution, a small increase in the D:N ratio occurred; however, when this experiment was repeated several days later with the same animal, no significant

change in the glucose elimination resulted. The small increase in the glucose output observed in the first experiment in which the acetate was injected intraperitoneally probably owes its origin to a breakdown of the glycogen still present in the dog. With phlorhizinized animals having a D:N ratio indicative of a complete diabetes, the administration of adrenalin or the production of lactic acid by muscular work or shivering may result in a considerable increase in the glucose excretion. This has been regarded as a resultant of the flushing out of the glycogen reserves since a repetition of the experiment is not followed by an increased glucose excretion. The condition in the present case is an analogous one, the shock of the first injection of the hypertonic salt solution in the peritoneal cavity probably causing the flushing out of the small amount of liver glycogen while the second administration was without such an effect.

In the twelve experiments which are summarized in Table II, no extra sugar was found in four cases, 4 to 16 per cent of the glucose equivalent was noted in four other instances, 32 per cent was eliminated in the first experiment on one animal and practically none on a second test. In only one dog was a significant amount of extra glucose excreted in more than one experiment, the results being 43, 18, and 23 per cent respectively after three successive injections. Nothing approaching a quantitative conversion was found in any of the twelve experiments and in the two instances in which there was an appreciable increase in the glucose excretion after the first injection of the acetate, later experiments resulted in only a small production of extra sugar. Controls showed that if glucose were injected or given orally an approximately quantitative excretion of the carbohydrate occurred in the urine.

In some cases the excretion of volatile acids was very uniform during the control periods while in other instances quite variable results were obtained. If one may calculate the extra volatile acid excreted after the injection of acetate as the excess found during the experimental period over the average of the first and second control periods, then the excretion of unchanged acetate usually does not exceed 15 per cent of that introduced. In the experiments with Dog 175, the extra acetate eliminated amounted to 4, 13, and 0 per cent respectively in the three experiments. In the case of Dog 120 there was less than 3 per cent excreted in the

first test. With Dog 169, 11 and 19 per cent of the injected acid were accounted for in the urine while with Dog 180 the increase in urinary volatile acid corresponded with 0 and 16 per cent of that administered in the second and third experiments. In general one may say that there is usually a small increase in the value of the urinary volatile acid after the injection of acetate and a fall following the introduction of glucose in phlorhizinized dogs. Since it is not converted to sugar and since it is not excreted to an appreciable amount, the obvious conclusion remains that the bulk of the acetic acid is oxidized in these animals. Experiments to determine the effect of the acetate administration on the respiratory quotient of phlorhizinized dogs will be carried out later.

TABLE III.

Effect of Subcutaneous Administration of 27 Gm. of Sodium Acetate on Urinary Volatile Acid Excretion of Normal Fasting Dogs.

Dog No.	Body weight.	Hourly excretion of urinary volatile acids.			Extra acetate excreted.	Remarks.
		Control period I.	Acetate period.	Control period II.		
	kg.	*mg.*	*mg.*	*mg.*	*per cent*	
177	14.6	61	71	45	3.7	
177		45	60	36	4.1	
41	14.2	55	32	36	0	
41		36	46	34	2.1	
175	10.4	19	40	27	2.9	Some $NaCH_3CO_2$ leaked from area of injection; included in urine sample.
175		27	28	27	0.2	

Effect of Injection of Sodium Acetate on Volatile Acid Excretion of Normal Dogs.—Six experiments were carried out on three normal fasting female dogs to determine the effect of the subcutaneous administration of acetate on the excretion of volatile acid in the urine. The results are summarized in Table III.

The normal dog is able to utilize nearly completely fairly large amounts of sodium acetate when introduced by the subcutaneous route. Only small increases in the urinary volatile acid excretion occurred after its injection, the maximum excretion of acetate amounting to only 4 per cent of that injected. The

normal dog must oxidize this molecule as does the diabetic animal. This agrees with the conclusions of Lusk (15) which indicate that acetic acid undergoes a rapid oxidation in the normal dog.

DISCUSSION.

If the theory that fat is capable of transformation into carbohydrate is cogent, and if acetic acid is its intermediary product, then this acid should also be a glucogenetic agent. We have demonstrated that such is not the case. There is no appreciable synthesis of glucose from sodium acetate when this substance is introduced subcutaneously or intraperitoneally in phlorhizinized dogs. Control experiments with the same animals showed that the ingested glucose was excreted practically quantitatively in the urine, which would indicate that had glucose been formed from the acetate, it would also have yielded extra urinary sugar.

It might be claimed that the amounts of sodium acetate given in our experiments were unphysiological. One might maintain that small quantities of acetic acid were readily changed into glucose but that when large doses are administered, the bulk of it escapes such a fate. There are several facts, however, which would seem to indicate that the above experiments were carried out with physiological amounts.

In the first place it would seem that if the body were overwhelmed by an excessive quantity of sodium acetate the excess would be excreted in large part in the urine unchanged—a condition which we have shown does not occur. Secondly, the amount of acetate given in the experiments reported here is no greater than that usually employed in tests with phlorhizinized dogs in which the glucogenetic action of various intermediates has been tested. In the latter experiments these amounts have never been considered too great to be normally metabolized. The experimental animals have usually been able to change any glucogenetic substances completely into glucose.

A further analysis indicates that the quantities of sodium acetate administered were within the physiological range. The experiments of Anderson and Lusk (16) offer the necessary data for such an analysis. These investigators report that a dog weighing 8.75 kilos oxidized as much as 7.65 gm. of fat hourly in doing a moderate amount of work on the 3rd day of fasting (Experi-

ment 28) at a time when the non-protein respiratory quotient was that of pure fat. This would correspond with the metabolism of 13.65 gm. of acetic acid per hour. On the basis of body weight this animal oxidized much more acetic acid than any of our animals were called upon to metabolize during the period of absorption which must have required at least several hours. The results of Anderson and Lusk represent a breakdown of sufficient fat to produce more than twice the amount of acetic acid given in many of our experiments. Therefore, the quantities of acetate administered by us must be considered well within the physiological limits either for testing the capability of the organism to oxidize it or for determining the ability of the animal to convert it to sugar.

SUMMARY.

The subcutaneous or intraperitoneal injection of sodium acetate into phlorhizinized dogs did not cause a significant change in the D:N ratio, a result which would indicate, contrary to the view of Geelmuyden, that this molecule is ineffective as a glucose former. These experimental data are offered as additional evidence that the animal is unable to transform a possible intermediate of the fatty acid portion of the fat molecule into glucose.

Only small amounts of the injected acetate escaped unchanged in the urine of the phlorhizinized or the normal fasting dogs as determined by the increase in the excretion of volatile acids. This suggests that the acetate molecule is practically completely oxidized in the living organism.

Sodium acetate acts as a violent purge when introduced orally.

BIBLIOGRAPHY.

1. Cremer, M., *Münch. med. Woch.*, 1902, xlix, 944.
2. Lüthje, H., *Deutsch. Arch. klin. Med.*, 1904, lxxx, 98.
3. Chambers, W. H., and Deuel, H. J., Jr., *J. Biol. Chem.*, 1925, lxv, 21.
4. Lusk, G., *Am. J. Physiol.*, 1908, xxii, 163.
5. Ringer, A. I., and Lusk, G., *Z. physiol. Chem.*, 1910, lxvi, 106.
6. Geelmuyden, H. C., *Skand. Arch. Physiol.*, 1920, xl, 211.
7. Thunberg, T., *Skand. Arch. Physiol.*, 1920, xl, 1.
8. Ringer, A. I., Frankel, E. M., and Jonas, L., *J. Biol. Chem.*, 1913, xiv, 539.
9. Clutterbuck, P. W., *Biochem. J.*, 1927, xxi, 512.
10. Schotten, C., *Z. physiol. Chem.*, 1882–83, vii, 375.

11. Thudichum, J. L. W., *Arch. ges. Physiol.*, 1877, xv, 12.
12. Dakin, H. D., *J. Biol. Chem.*, 1907, iii, 57.
13. Deuel, H. J., Jr., Wilson, H. E. C., and Milhorat, A. T., *J. Biol. Chem.*, 1927, lxxiv, 265.
14. Gaebler, O. H., personal communication.
15. Lusk, G., *J. Biol. Chem.*, 1921, xlix, 453.
16. Anderson, R. J., and Lusk, G., *J. Biol. Chem.*, 1917. xxxii, 421.

THE TRIPARTITE NATURE OF VITAMIN B.

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This paper is partly concerned with confirmation of the existence of two components of vitamin B, which are already widely regarded as distinct, but principally deals with a third hitherto unrecognized substance which has been involved in much of the past experimentation on vitamin B. The first of the three factors or substances is the relatively thermolabile antineuritic vitamin; the second is the relatively stable factor which Goldberger and others have associated with the prevention of pellagra; the third is highly thermolabile, and so far has been associated only with the maintenance of weight and general condition of adult pigeons. We have arrived at our conclusion as to the threefold nature of vitamin B by a series of rigid experimental comparisons of the dietary requirements of growing rats and adult pigeons.

Since the preparation of our preliminary paper (1) some further evidence has accumulated from many sources as to the dual nature of vitamin B. Chick and Roscoe's review (2) of the subject has been amplified somewhat by Sherman and Axtmayer (3). Both of these papers contain references to the principal previous literature. In addition to the references noted by these authors, mention may well be made of the work of Hassan and Drummond (4) who used heat and alkali for the destruction of the labile factor to permit detection of the stable one, and Macy, Outhouse, Long, and Graham (5), who showed a qualitative difference in human milk, cow's milk, and wheat embryo as to vitamin B content. All of the foregoing work is in general agreement as to the existence of the second factor of vitamin B as well as the antineuritic one. This second factor of vitamin B is readily demonstrable with rats, but not with pigeons, as shown by Seidell (6).

In the following pages we will present further evidence as to the second factor of vitamin B, verifying the conclusion that it is potent for rats, but not noticeably so for pigeons, and in addition will show that there is a third factor of vitamin B potent for pigeons but apparently not for rats.

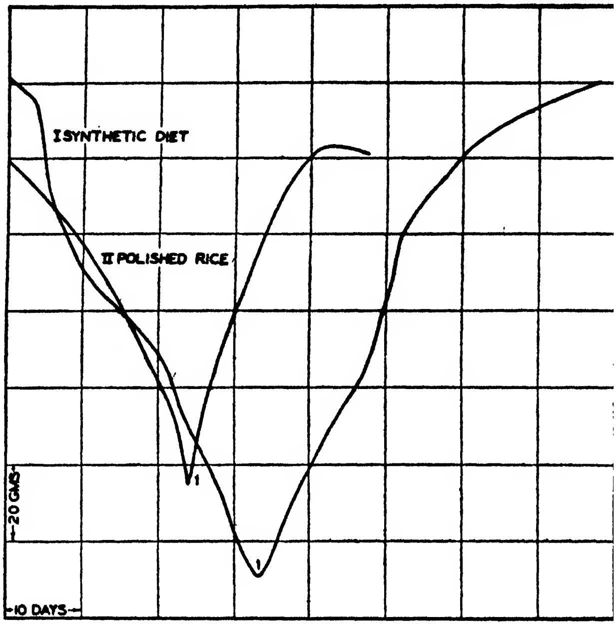

CHART I. Comparison of synthetic diet and polished rice as basal diets for pigeons. At point 1 a supplement of 1 gm. of dried brewers' yeast every other day was begun. Curve I represents average of four birds, Curve II of three birds, the birds in each group showing closely consistent behavior. Supplements were given in gelatin capsules.

Materials of Experimentation.

For the experimental work with rats we used the basal diet (No. 107) of Sherman and Spohn (7), consisting of extracted casein 18, salt mixture 4, butter fat 8, starch 68, cod liver oil 2. We are by no means convinced that such a diet as this is free from unrecognized vitamin factors (8) or even quite free of vitamin B, but it has the merit of being well standardized and possessing certain definite deficiencies.

As a diet for pigeons, we have used polished rice *ad libitum* in most instances, but have also in a number of cases used certain lots of the above Sherman and Spohn diet, which were also tested in parallel experiments with rats. The synthetic diet was also fed *ad libitum* to the pigeons, as forced feeding is impracticable for rats and we wished to maintain strict comparability in our experiments on the two species to bring out any differences in their dietary requirements. Under our conditions of experiment we were unable to note any significant differences between the synthetic diet and polished rice in the nutrition of adult pigeons. Chart I shows some typical results. Other instances appear in the other charts.

As a source of the second factor of vitamin B, which is characterized by a high degree of thermostability, we used brewers' yeast, autoclaved for 6 hours at 121°, substantially according to Smith and Hendrick (9). Our experience teaches us that this operation must be carried out carefully, in small containers, to insure heating through and through. Excessive heating in a dry condition is apparently capable of destroying both factors but we are not able to make a final report on this phase of the subject yet.

As a source of the antineuritic factor of vitamin B we have used principally a special form of fullers' earth preparation of brewers' yeast, which was developed in the course of attempts to isolate the antineuritic vitamin. This product is made by bringing a limited amount of fullers' earth into contact with yeast extract with the intervention of a collodion membrane. The details of the method are given below, though they are not essential to the main theme and may be omitted by the reader who has no special interest in that phase. Such preparations for brevity are designated by "Y", followed by a serial number. The letters which in certain cases follow the serial number indicate that successive portions of fullers' earth were used to treat a given quantity of extract; *e.g.*, A represents a second portion of fullers' earth, B a third portion, *etc.*

Method of Making Y Preparations.

It had been noted that in treating a given quantity of extract by direct contact with successive small portions of fullers' earth, the

second portion was, weight for weight, equal or superior to the first in activity for pigeons and, per unit of nitrogen, almost twice as high in activity. We believed this to be due to contamination and consequent loss of selectivity of the adsorbing surfaces of the first portion of fullers' earth by proteins or basic substances of high molecular weight which might be held back by a suitable membrane. To test this hypothesis and, if possible, obtain a fullers' earth of maximum physiological activity and minimal contamination with inactive organic material, we have experimented at length with dialyzing membranes. To permit their use in connection with large quantities of foodstuffs, or extracts thereof, we devised, after many discouraging trials, the apparatus shown in Fig. 1.

The apparatus consists of the following parts: A 2 foot length of vitrified drain tile of 8 inches internal diameter is fitted with countersunk 12 inch diameter circular heads of 2 inch board, the depressions in the boards being lined with soft rubber sheets to serve as gaskets. The two heads are pierced by six $\frac{1}{4}$ inch tie rods running outside along the length of the tile and distributed equidistantly around its circumference. The tie rods are threaded at the ends and fitted with wing nuts for easy opening and closing of the vessel. Each such container is provided with an internal cylindrical vessel $5\frac{1}{4}$ inches in diameter and $21\frac{1}{2}$ inches long, placed concentrically within it and held in place by a central shaft, the ends of which fit into depressions at the center of the inside of each wooden head of the outer vessel. This inner vessel is constructed entirely of monel metal, the walls of the cylinder being of wire gauze of 24 meshes per inch, silver-soldered evenly to the edges of the solid end plates. In operation, the outer vessel, with its concentric inner vessel in place, is laid on two horizontal and parallel motor-driven wooden rollers of 4 inch diameter, with which the circular wooden heads of the outer vessel make frictional contact (see Fig. 1). The speed of rotation secured by chain drive and motor is 5 to 10 revolutions per minute.

For use the gauze walls of the inner vessel are coated with collodion, applied by rotating the cylinder once horizontally while its lower wall dips in a U. S. P. collodion solution diluted with half its volume of 1 to 3 alcohol-ether mixture. This operation requires some practice, and at best the thickness and permeability of the

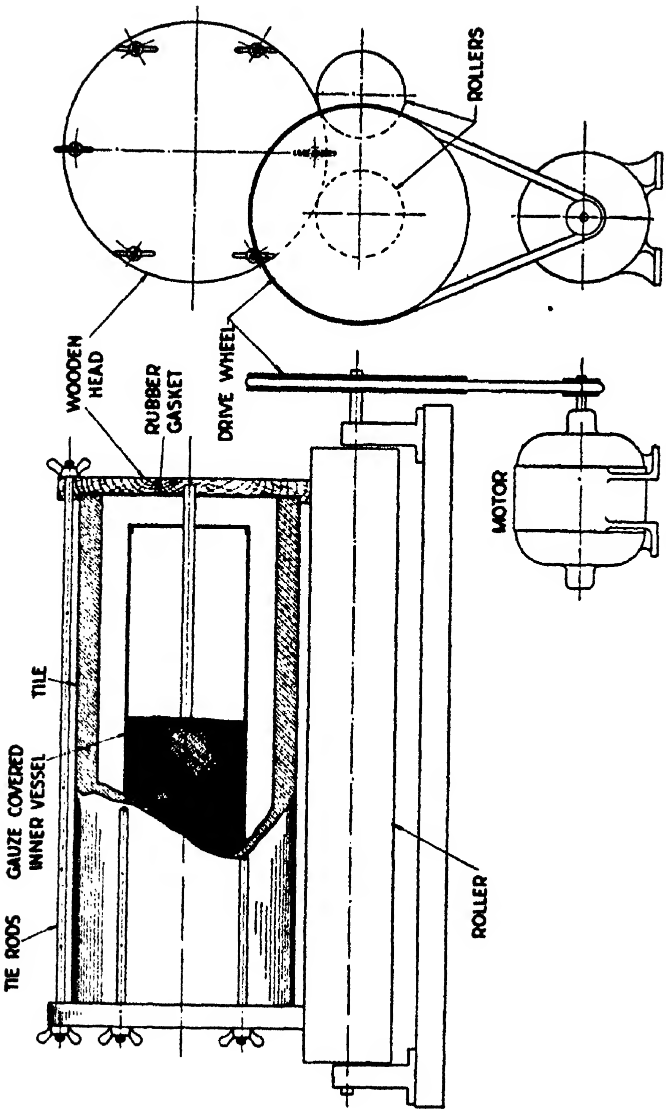

FIG. 1. Dialysis and adsorption apparatus.

membrane cannot be controlled as satisfactorily as desired. If too thick from rotating more than once, or in too strong a solution, diffusion will be prohibitively slow. If made too thin it will be apt to show holes at the outset or rupture in operation. In our practice a single rotation in a solution of suitable dilution is followed by a 1 minute drying, accomplished by removal from the solution and rotation horizontally in the air. The cylinder is then gently submerged in water and after an hour or two tested by blowing through the filling hole, provided in the head of the cylinder and fitted with a rubber stopper and convenient tube. A good pair of lungs will detect any holes of significant size. If the holes are not numerous they may be patched by drying the area immediately about them with a soft linen cloth and painting them over with U. S. P. collodion.

The collodion-coated vessel is now ready for filling. It is first placed in position in the tile cylinder, the latter being stood on end on its wooden head. Through the filling hole 20 per cent methanol, containing the desired amount of suspended fullers' earth, is poured into the coated cylinder to three-fourths its capacity. At the same time the tile outer cylinder is filled with the crude material. We have used rice polish or killed yeast made up to a fairly thick gruel with dilute methanol, or yeast extract containing methanol. The inner vessel is now stoppered, the outer one closed by bolting on the upper head in place, and the contrivance put on the rollers to be continuously rotated for from 2 to 4 days. Methanol is as good a preservative as ethanol and much less expensive.

At the conclusion of the operation the contents of the two vessels are siphoned off separately. The fullers' earth left in the inner vessel is washed out and collected on a filter, along with that in the liquid siphoned off from that vessel.

The contents of the outer vessel may be discarded or may be further extracted with a new charge of fullers' earth, the filtrate from the first fullers' earth being used to charge the inner cylinder. Before the inner cylinder is used again, however, the membrane is burned off and renewed, because after a few days use it becomes plugged with colloids, *etc.* The cylinder is first dried thoroughly to permit burning off the old collodion in a carefully applied gas flame, is brushed vigorously to remove charred matter, and recoated with a new membrane. The collodion membrane itself

adsorbs an amount of vitamin that is detectable but negligible in comparison with the total. We have used this apparatus extensively with brewers' yeast, but found it most economical to utilize an extract made substantially after the Osborne-Wakeman method and concentrated *in vacuo* to about 10 per cent total solid content. Such an extract was used in making the Y preparations described herein and was supplied us by the Harris Laboratories of Tuckahoe, New York, whose cooperation we are pleased to acknowledge here.

In general, it may be said that by using fullers' earth in the proportion of 1 part to 100 parts of rice polish or dry brewers' yeast involved, we obtained activated fullers' earth by this process which supplies the full antineuritic complement to rats or pigeons in doses of from 5 to 10 mg. per day per animal over several weeks feeding period. A second fullers' earth charge of equal amount dialyzed against the residual rice polish or yeast extract contains about half the activity per unit of weight of the first portion. In one case of yeast extraction activity was still detectable in the fifth successive charge of fullers' earth. The nitrogen content of successive charges progressively declines from the initial figure of 2 to 3 per cent. The samples of fullers' earth are almost Ca-free if highly activated, though 3.2 per cent of Ca is present in the original earth. Solutions of alkaloidal salts, such as quinine sulfate, also displace Ca from fullers' earth as the alkaloid is adsorbed.

These Y preparations when also supplemented by autoclaved yeast are more active, weight for weight, for rats than the usual fullers' earth preparations made by direct contact of adsorbent with extract (Chart II, Curves I and II). When used as the sole supplement for pigeons a similar difference is observable. They also have some advantage in being more free from substances of physiological importance other than the antineuritic factor. This is shown by an experiment with rats indicated in Chart II, Curves III and IV. It will be noted that the Y preparation, when used as the sole supplement, was scarcely more effective in quadrupled doses than in the smaller doses, while a fullers' earth, S 88, treated in the same way, except for the omission of the collodion membrane, betrayed the presence of some second useful factor (the thermostable one) when the dose was quadrupled.

Supplementary Relationship of Activated Fullers' Earth and Autoclaved Yeast.

The above materials serve to show in a very striking manner the supplementary relation for rats between the antineuritic factor in

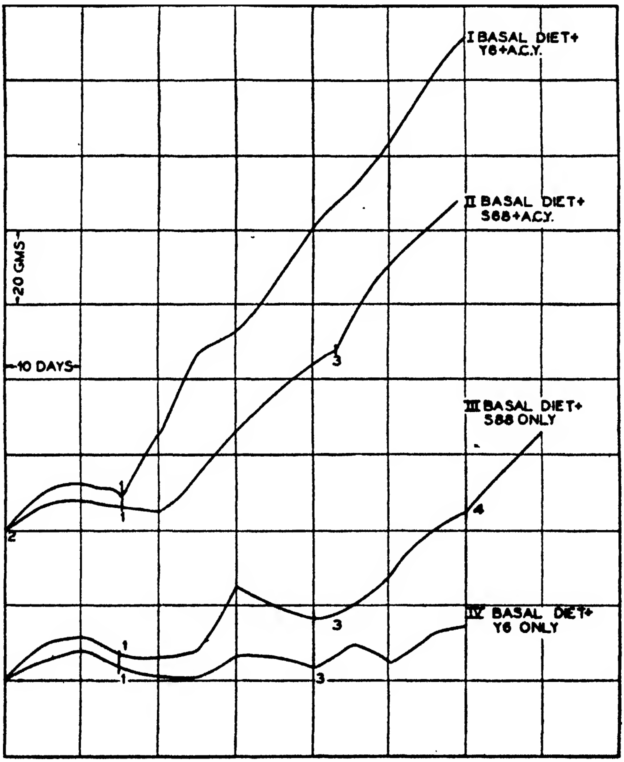

CHART II. Effect on growth of rats of fullers' earth preparations activated with and without the use of a dialyzing membrane. At point 1 a supplement of 10 mg. of fullers' earth preparation daily was begun, and at point 2 a daily supplement of 1 gm. of autoclaved yeast (A. C. Y.). At point 3 the daily supplement of fullers' earth preparation was increased to 40 mg. and at point 4 to 80 mg. Supplements were mixed with a small portion of the diet which was fed first to insure complete consumption.

the form of Y preparations and the thermostable factor as supplied by autoclaved yeast. The curves shown in Chart III show the behavior of each type of preparation alone and in combination, and

leave no doubt as to the dual nature of vitamin B, since they are typical of scores of records we have obtained in which fifteen or twenty distinct Y preparations have been involved.

When the same or similar Y preparations are added as supplements to the diet of pigeons receiving polished rice or the identical synthetic diet used for rats, decline in weight is stopped, as shown

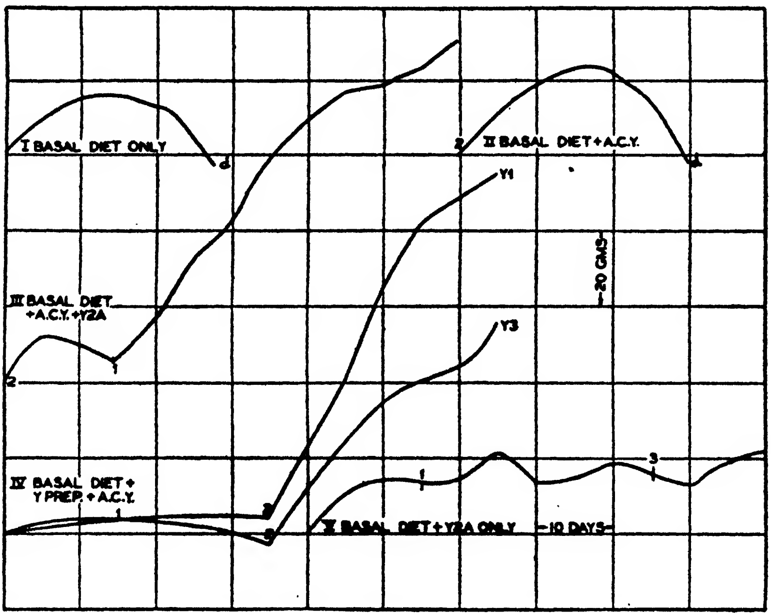

CHART III. Growth of rats affected by two vitamin B factors. At point 1 a supplement of 10 mg. daily of Y preparation was begun; at point 2 a supplement of 1 gm. of autoclaved yeast (A. C. Y.) was begun; at point 3 a supplement of 40 mg. of Y2A was begun. All curves are typical of a group. Supplements were mixed with a small portion of the diet which was fed first to insure complete consumption.

in Chart IV. A temporary sharp rise often occurs (Curves III and V) and in the case of large doses a prolonged gradual rise may sometimes be observed (Curves IV and V), but the sharp, rapid, and continuous rises to the normal level of weight, such as are characteristic of brewers' yeast addenda (see Chart I) or a whole wheat diet as in the last period of Curves III and V, Chart IV, do not result from the use of the Y preparations. A subnormal level

may be maintained for many weeks, a condition not very different from that of the rat when receiving the same material as the sole vitamin B addendum (see Chart III, Curve V). But in the case of pigeons, the addition of autoclaved yeast has no effect (compare

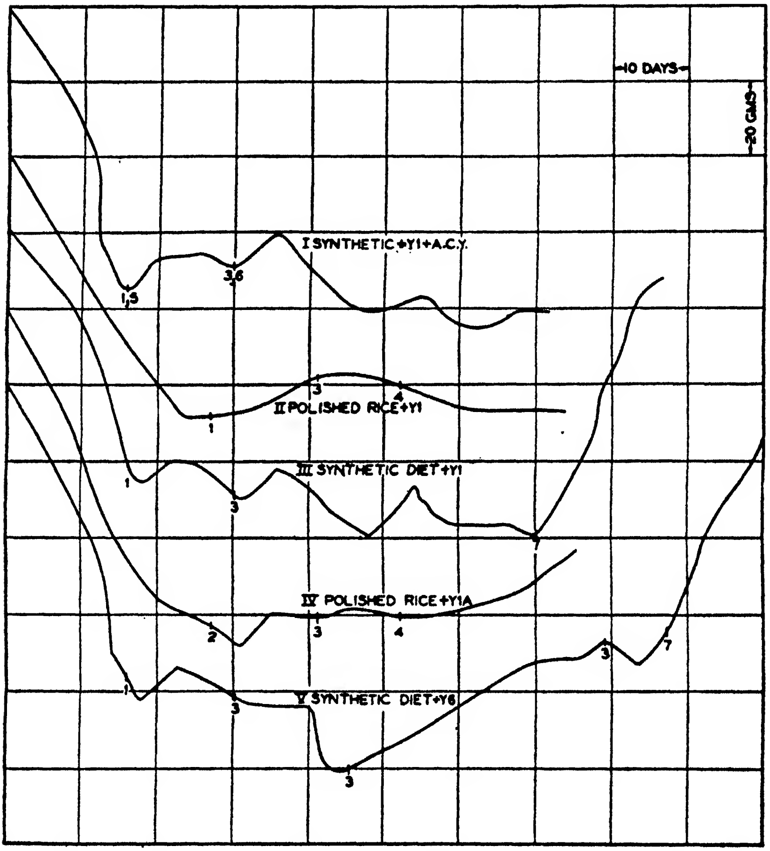

CHART IV. Maintenance of pigeons affected by two vitamin B factors. At point 1 a supplement of 20 mg. of Y preparation was begun; at point 2, 50 mg.; at 3 the previous dose was doubled; at 4 the last previous dose was quadrupled; at 5 a supplement of 1 gm. of autoclaved yeast (A. C. Y.) was begun; and at 6, 2 gm. of autoclaved yeast (A. C. Y.). At point 7 the diet was changed to whole wheat without addenda. Each curve represents the average of a group of three or four birds, the birds in each group being consistent in reaction to diet changes. Supplements were administered every other day in gelatin capsules.

Curves I and III, Chart IV). These findings are entirely in harmony with those of Seidell (6), who used a more elaborately purified yeast fraction as a source of the antineuritic factor. He did not call attention to the failure of his preparation to restore the normal weight of pigeons, though this fact is shown by his experimental results. In our experience birds in this state of incomplete maintenance always exhibit ruffled feathers, subnormal appetite, and a considerable degree of lassitude.

Evidence of a Third Factor of Vitamin B.

The above data show that pigeons, which have been brought to a subnormal level on the synthetic diet supplemented by special fullers' earth, respond promptly when the diet is changed to whole wheat (see Chart IV) and soon attain a normal weight level. The same is true when polished rice is used as basal diet, though for brevity such a curve has been omitted. The appearance of the birds simultaneously undergoes a marked improvement as to condition of the feathers, appetite, and general activity. These changes might be ascribed to any one of several factors, such as mineral constituents or better quality of protein in the whole wheat.

However, Chart I shows that the same improvement occurs when 1 gm. of air-dried brewers' yeast is given every other day, while the basal diets remain unchanged. Since this result is not obtained with autoclaved yeast or Y preparation, or both, as supplements, the improvement in weight and condition of the birds must be due to a thermolabile factor originally present in the yeast, which is different from the antineuritic factor extracted from the yeast by fullers' earth and present in abundance in these Y preparations.

It might be supposed that this factor was vitamin A or D but for the conclusive fact that the synthetic diet containing butter fat and cod liver oil, for the purpose of supplying these factors, showed no superiority over polished rice. There still remained a slight question that the factor sought might be vitamin E, because butter fat is not a very rich source of vitamin E. But this was answered in the negative, for we fed 0.5 to 1.0 gm. of ether extract of wheat bran as a further supplement to polished rice and Y preparation without marked benefit.

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BIBLIOGRAPHY.

1. Williams, R. R., and Waterman, R. E., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 1.
2. Chick, H., and Roscoe, M. H., *Biochem. J.*, 1927, xxi, 698.
3. Sherman, H. C., and Axtmayer, J. H., *J. Biol. Chem.*, 1927, lxxv, 207.
4. Hassan, A., and Drummond, J. C., *Biochem. J.*, 1927, xxi, 653.
5. Macy, I. G., Outhouse, J., Long, M. L., and Graham, A., *J. Biol. Chem.*, 1927, lxxiii, 153. Macy, I. G., Outhouse, J., Graham, A., and Long, M. L., *J. Biol. Chem.*, 1927, lxxiii, 189.
6. Seidell, A., *Bull. Soc. chim. biol.*, 1926, viii, 746.
7. Sherman, H. C., and Spohn, A., *J. Am. Chem. Soc.*, 1923, xlv, 2720.
8. Palmer, L. S., and Kennedy, C., *J. Biol. Chem.*, 1927, lxxiv, 591; 1927, lxxv, 619.
9. Smith, M. I., and Hendrick, E. G., *Pub. Health Rep., U. S. P. H. S.*, 1926, xli, 201.

A STUDY OF THE EFFECT OF INSULIN UPON THE SUGAR CONTENT OF ERYTHROCYTES, INCLUDING A COMPARISON OF THE DIRECT AND INDIRECT METHODS OF MEASUREMENT.*

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(Received for publication, April 30, 1928.)

It has been reported recently that insulin causes variations in the glucose content of the erythrocytes of the dog, the rabbit, and man that are disproportionate to the concomitant changes in the whole blood. In the course of an investigation with reference to the cause of the condition known as hypoglycemia, Foshay (1) observed several instances where the intracorpuscular glucose dropped rather suddenly and markedly even to hypoglycemic levels while the concentration in the plasma and the whole blood still remained high. Rona and Sperling (2) also have made similar observations. In addition they have noted occasional occurrences of corpuscular hypoglycemia in the midst of alimentary hyperglycemias without any insulin having been administered. Both Foshay and Rona and Sperling have recorded experiments where the intracorpuscular sugar at times exceeded considerably that in the plasma and in general fluctuated rather sharply within short intervals. These experiences are in line with those reported somewhat earlier by Wishart (3). This investigator administered glucose in various ways to animals in a variety of nutritional conditions and concluded that the sugar content of the red blood corpuscles under such circumstances is subject to considerable variation and irregularity. Taken altogether the work of these different investigators might be interpreted to signify that the sugar of the erythrocytes is capable of changes that are at least partially independent of the plasma sugar concentrations.

Recorded in the literature, however, are some observations which seem to be in opposition to the view just outlined. Michaelis and Rona (4) have shown that the sugar of the blood is in a free and diffusible form. Ege, Gottlieb, and Rakestraw (5) have found that at body temperature added glucose distributes itself almost instantly between plasma and corpuscles. These observations suggest that the sugar of the corpuscles and of the

* Aided in part by a grant from the De Lamar Mobile Research Fund of the Harvard Medical School.

plasma should tend to vary at more or less the same rate and that the tendency should be to maintain equilibrium at all times. And in fact Folin and Berglund (6) have found that after the ingestion of glucose by normal individuals the sugar content of the corpuscles changed in a manner nearly parallel to that of the plasma. Hansen (7) has obtained similar results with normal and with diabetic patients.

It is apparent that the situation with regard to the behavior of the sugar of erythrocytes has discrepancies which should be clarified. It seemed to us that the methods of measurement employed might be responsible for the discordant results which have been reported. Therefore, it appeared desirable that these should receive further scrutiny, especially since the determination of the changes of the intracorpuscular glucose has been suggested as a means of forecasting hypoglycemic reactions.

Experimental Procedure.

There are two general procedures available for determining the sugar content of the corpuscles. In one, a portion of a blood sample is taken for a determination of the sugar in the whole blood, while the remainder is centrifuged in order that the plasma may be separated from corpuscles and its sugar content measured. If the volume percentage of red cells is known then the sugar concentration of the corpuscles can be computed by use of the formula:

$$\frac{\text{Whole blood sugar} - (\text{plasma sugar} \times \text{per cent plasma})}{\text{Per cent corpuscles}} = \text{concentration}$$

of sugar in corpuscles.

It is apparent that this method has important possibilities for yielding results which may be quite at variance with the true situation. The errors of measuring the small quantities of blood and of solutions used and the determination of small quantities of sugar may all be thrust upon the value calculated for the sugar concentration of the erythrocytes. A consideration of two concrete cases will make this clear. (*Cf.* table on p. 325.)

In Case 2 a decrease of only 4 mg. per cent in the whole blood sugar when combined with a similar increase in the plasma sugar—variations which are within the limits of analytical methods—is quite sufficient to cause a much larger decrease in the value assigned to the sugar of the erythrocytes. Even with the normal

value of 45 per cent of erythrocytes the possible error is important, and with the lower value of 27 per cent of erythrocytes (a value not as extreme as some to be found in the literature of this subject) the error is much increased. When the quantity of sugar in the whole blood and plasma is nearly the same, and when the per cent of cells and plasma approach equality, the opportunity for error by the indirect method is at a minimum. As the data actually obtained in experiments diverge in either of the possible ways from this ideal situation then the probability that the calculated value may be erroneous is greatly enhanced.

Case No.	Whole blood sugar.	Plasma sugar.	Volume of erythrocytes.	Sugar of corpuscles (calculated).
	mg. per cent	*mg. per cent*	*per cent*	*mg. per cent*
1	110	125	45	91
			(27)*	(69)
2	106	129	45	77
			(27)	(44)

* Values in parentheses indicate the effect of lower erythrocyte volume.

The second general procedure is to separate the corpuscles and then determine their sugar content directly. The difficult point here concerns the completeness with which the plasma can be removed from the corpuscles. Since the plasma usually has the higher sugar content it is obvious that failure to accomplish complete separation will yield values higher than the real sugar content of the corpuscles. Also it has been stated that it is difficult to precipitate all of the protein from the corpuscles (8). However, Wu (9) has reported some analyses which indicate that this can be accomplished satisfactorily. A third objection (8) which has been made to this method is that glycolysis occurs rapidly in the sediment and leads to a considerable lowering of the sugar concentration. This point will be considered again.

In view of the uncertainties which are inherent in each method, we realized at the start of our experiments that it would be unwise to rely upon results obtained by either of the above procedures alone. Therefore, we have determined the sugar concentration of the corpuscles by applying both methods simultaneously to every sample analyzed.

Methods.

Collection and Manipulation of Blood Samples.—Blood was drawn through a hollow needle into a syringe containing a few mg. of heparin as anticoagulant. In the experiments upon dogs it was sometimes permitted to flow directly into a tube containing the powdered anticoagulant. The tube was stoppered and inverted twice to insure thorough mixing. About 1 cc. of blood was poured upon a small watch-glass and one of us immediately filled two hematocrit tubes and started the determination of the percentage volume of erythrocytes in the sample. Meanwhile another operator pipetted out a sample of whole blood and proceeded with the preparation of the protein-free filtrate. At the same time, a third operator placed the remainder of the blood in a high speed centrifuge for separation of the cells and plasma. The centrifuge and hematocrit were allowed to run for 10 minutes. At the end of this period a portion of the plasma was drawn off through a capillary-tipped siphon into another glass tube and used for preparation of the plasma filtrate. Meanwhile, one of us continued to siphon off the plasma from the cells removing also the so called buffy coat and sacrificing the top portion of cells to insure that the removal of plasma should be as complete as possible. The erythrocytes were drawn up into a Folin blood pipette, measured while the meniscus was observed against a strong light, and then transferred with slow drainage to a small Erlenmeyer flask. The water for laking the cells was added to the flask and then drawn up into the pipette three successive times to lake the cells adhering to its interior surface and to wash out their soluble contents.

The person or animal furnishing the blood samples, the centrifuge, the hematocrit, and apparatus for preparing the protein-free filtrates were all brought together in the same room (except with one coma case). Usually all manipulations incident to the preparation of the three filtrates were accomplished within 15 minutes after drawing the blood. The sugar determinations were begun as soon as several samples of blood had been taken and all measurements were completed as soon as possible after the end of the experiment. The only exception was in one long experiment terminated late at night, and in this case the filtrates were placed in a refrigerator at 0°, as soon as obtained, and allowed to stand until the next morning.

Analytical Methods.—All blood specimens were deproteinized by the Folin-Wu (10) method, with the modifications that for the plasma samples $\frac{1}{3}$ volume each of sodium tungstate and $\frac{2}{3}$ N sulfuric acid were used while for the erythrocytes 2 volumes each of tungstate and acid were employed. In each case the final dilution was to 10 times the original volume. The Folin-Wu method was used for the sugar determinations. Glucose from the Bureau of Standards was employed for preparation of standard solutions.

Measurement of Percentage Volume of Cells.—A Bausch and Lomb capillary tube hematocrit capable of more than 5000 revolutions per minute when loaded was employed throughout. It was allowed to run for a period of 10 minutes before our readings were taken. Van Allen (11), after a careful study of hematocrit methods, has found that when the speed of revolution is in the neighborhood of 3600 per minute that rotation for 9 to 11 minutes is sufficient to produce constant readings. Measurements upon each sample were made in duplicate and usually agreed within 1 unit. Where there was a divergence the average value was used in making our calculations.

Separation of Corpuscles from Plasma.—This was accomplished by rotation for 10 minutes in a centrifuge capable of more than 3000 revolutions per minute when loaded. In preliminary experiments it was found that 10 minutes rotation was sufficient to pack the corpuscles to a minimum volume. Also it was determined that a shorter period of centrifuging (1 minute) did not give any lower values for corpuscle sugar when measured by the indirect method.

Anticoagulant.—In preliminary experiments, oxalates and heparin were compared for this purpose. With heparin it was found that hemolysis could be completely avoided so it was used throughout the remaining experiments; 1 to 2 mg. of the solid substance was sufficient for 10 cc. of blood. Its effect upon the Folin-Wu reagents was tested and found to be negligible at the concentrations employed.

Fermentation Experiments.—To one-eighth of a cake of freshly washed yeast[1] (Fleischmann) in a centrifuge tube, 10 cc. of tungstic

[1] The yeast had been washed three times with distilled water. A control test on the washed yeast gave no appreciable blank with Folin-Wu reagents. The advantages of washed yeast have recently been reported by Somogyi (12).

TABLE I.

Experiments upon Diabetic Patients.

Blood sample No.	Time.	Volume of erythrocytes.	Sugar concentrations. Whole blood.	Plasma.	Erythrocytes. Directly measured.	Computed.	Remarks.
Experiment 1, F. G.							
	a.m.	per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	
1	9.50	45, 45	420	430	408	407	Fasting.
	10.00	Insulin, 40 units subcutaneously.					
2	10.30	45, 45	348	373	295	317	
3	11.10	46, 47	216	220	208	211	
4	12.15	45, 45	88	84	78	93	
5	1.12	45, 46	43	45	44	40	Mild hypoglycemic reaction occurred.
Experiment 2, J. L. McL.							
1	8.40	45, 46	230	244	212	213	Fasting.
	8.55	Insulin, 24 units subcutaneously.					
2	9.55	46, 46	184	200	160	165	
3	10.55	46, 47	143	147	135	138	
4	11.55	45, 46	49	40	57	59	
	12.15	Hypoglycemic reaction took place.					
Experiment 3, J. L. McL.							
1	8.45	46, 47	216 12* (204)†	246 6* (240)	182 13* (169)	181 18* (162)	Fasting.
	9.00	Insulin, 24 units subcutaneously.					
2	10.00	46, 47	198 10* (188)	220 7* (213)	168 14* (154)	172 13* (159)	
3	11.00	46, 47	137 13* (124)	154 7* (147)	122 18* (104)	117 19* (97)	
4	12.00	45, 46	59 13* (46)	62 7* (55)	54 16* (38)	55 20* (35)	

* Non-fermentable reducing substance.

† The numbers in parentheses represent the fermentable sugars.

acid filtrate were added. After thorough mixing of the contents, the tubes were placed in a water bath and allowed to stand for 10 minutes at 37–40°. Then the yeast was thrown down by the aid of the centrifuge and the reducing substance in the supernatant fluid was determined by the Folin-Wu method. In measuring the weak colors obtained, the directions of Folin and Svedberg (13) were followed. For a discussion of methods for determining fer-

TABLE II.

Gabr. Ab. In diabetic coma at beginning of experiment.

Blood sample No.	Time.	Volume of erythrocytes.	Sugar concentrations.				Insulin.	
			Whole blood.	Plasma.	Erythrocytes. Directly measured.	Erythrocytes. Computed.	Time.	Units.
	p.m.	*per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*p.m.*	
1	12.10	42	650	688	600	597	12.05	30
2	2.10	40, 42	640	656	616	617	12.45	20
							1.20	20
3	3.25	45, 46	588	628	516	540	2.15	30
							3.05	40
4	4.35	41, 42	472	480	448	461	4.00	40
5	5.30		420				5.00	40
6	6.50	42, 43	372	400	254	334		
7	8.50	41, 42	316	332	292	293	7.30	20
8	9.55	40, 42	286	286	256	286	9.30	40
9	10.55	40, 42	228	218	228	242	10.00	20
	a.m.							
10	12.00	40, 40	198	196	172	201	11.00	10
							a.m.	
11	1.23	40, 40	176	174	154	179	12.40	10

mentable sugars reference should be made to the recent work of Benedict (14) in which some hitherto unrecognized errors are pointed out.

Results.

The changes of the sugar content (total reducing substance) of the whole blood, plasma, and erythrocytes, which take place simultaneously in these different portions of the blood under the influence of insulin, are presented in Tables I to III. Some ex-

TABLE III.

Experiments upon Diabetic and Normal Dogs.

Blood sample No.	Time.	Volume of erythrocytes.	Sugar concentrations. Whole blood.	Plasma.	Erythrocytes. Directly measured.	Computed.	Remarks.
			Experiment 5; pancreatectomy; Dog 1.*				
	a.m.	*per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	
1	9.50	44, 45	274	408	132	106	Fasting.
	10.20	Insulin, 20 units intravenously.					
2	10.32	44, 45	252	366	120	109	
3	10.58	43	165	202	104	116	
4	11.38†	47, 48	83	88	82	77	
			Experiment 6; pancreatectomy; Dog 2.‡				
1	11.00	40, 40	274	373	133	125	Fasting.
	11.05	Insulin, 40 units subcutaneously.					
2	11.25	36, 36	276	322	128	194	
3	11.49	36, 36	221	300	118	80	
	p.m.						
4	2.06	43, 43	52	41	61	66	Animal shivering.
5	3.43	41, 41	34	29	30	42	
	5.20						Convulsions and collapse.
6	5.30	40, 41	48	36	29	65	
7	5.45§	39, 38	68	77	34	53	
			Experiment 7; normal; Dog 2.¶				
	a.m.						
1	11.35	36, 37	82	96	63	57	Fasting.
	p.m.						
	12.10–12.21	20 gm. glucose in 100 cc., intravenously.					
	12.27	Insulin, 36 units subcutaneously.					
2	12.30	35	236	296	111	124	
3	1.00	41, 42	100	122	67	69	
4	1.47	46, 47	61	58	53	64	
5	4.12	38, 37	44	40	44	51	
6	4.51‖	41	44	33	42	59	

* Weight 16 kilos; operation 2 days preceding experiment.

† At 11.36, the dog made several convulsive movements, but remained fully conscious and was able to drink the sugar solution offered at 11.44.

‡ Weight 18.4 kilos; operation 4 days preceding experiment.

§ At 5.34 a solution of cane sugar in milk was given by stomach tube.

¶ Weight 19.5 kilos.

‖ At 4.28 the dog showed excessive salivation and muscular weakness; at 4.50 urinary incontinence and convulsions; at 5.00 was given sugar solution by stomach tube; recovery complete.

periments upon diabetic patients are given in Tables I and II. All had high initial blood sugars (one was in a coma) and in each case this was markedly and rapidly reduced. In the first two instances the drop was permitted to continue until a hypoglycemic reaction was obtained. Table III presents the results of experiments upon dogs. In two instances the animals had been depancreatized. One received insulin subcutaneously and the other intravenously. In each case the blood sugar fell from about 300 mg. per cent to convulsive levels. We also include one experiment (Table III, Experiment 7) upon a normal dog which received an intravenous infusion of glucose followed by insulin.

In addition we have five other experiments upon human diabetics (two of whom had hypoglycemic reactions), four more experiments upon diabetic dogs, and seven upon normal ones, with and without glucose administration. Inasmuch as the results in all cases are similar and entirely in accord with those presented here it does not seem necessary to record all of them.

In several of the experiments we have determined the non-fermentable as well as the total reducing substance in the various portions of the blood. Table I, Experiment 3, presents the results of a typical case before and after correction for non-fermentable material has been applied. It will be noted that the agreement between the directly measured and the computed values for erythrocyte sugar is practically as good whether the corrected (fermentable sugar) or uncorrected values are considered. Especially is this true when it is remembered that making the correction introduces the errors of several additional measurements into the already highly involved process for determining erythrocyte sugar by the computation method. Since the uncorrected and the corrected values both present the same fundamental picture with regard to the changes of erythrocyte sugar it is simpler and probably almost as accurate to consider only the total reducing substances as we have done in the other tables.

Incidentally it may be noted by inspection of Table I (Experiment 3) that the effect of insulin upon the concentration of non-fermentable substances in the various portions of the blood is very slight. In fact the variations are so small that they may be explained by the difficulties involved in obtaining the measurements.

DISCUSSION.

At the beginning of this work it was felt that it would be unsafe to rely upon the results obtained by either the direct or the indirect method alone in view of the deficiencies that are inherent (see the section "Experimental Procedure") in each of these processes for measuring the sugar content of erythrocytes. Therefore, it was decided to apply both procedures conjointly to each sample of blood analyzed in the hope that at least the approximate limits of the corpuscle sugar concentration might be marked out with definiteness sufficient to enable us to follow its variations under the influence of insulin. This expectation has been quite satisfactorily fulfilled. It may be noted that for about 65 per cent of the analyses recorded in Tables I to III the values obtained by the direct and by the indirect methods do not diverge from each other by more than about 10 per cent. And there are a number of other instances where the agreement does not come within the arbitrary limit yet is sufficiently close so that there is little difficulty in deducing the sugar content accurately enough to follow the trend of the changes taking place. Including these we may safely say that for more than 80 per cent of the blood samples analyzed the concentration of sugar in the red blood corpuscles is known fairly definitely. In none of these cases do we encounter any suggestion that insulin causes a sudden and a marked drop in the sugar of the corpuscles while that in the plasma and whole blood remains at a high level. Rather our results when taken in the aggregate indicate that in human beings the sugar content of the various portions of the blood falls off at about the same rate under the influence of insulin and that the tendency is to maintain an equilibrium between the corpuscles and the plasma which never falls far out of balance. The only parts of our data which might appear to conflict in any way with this interpretation are those instances in which the value of the corpuscle sugar is somewhat uncertain because the two methods for its determination have yielded quite discordant results. In considering the significance of these instances, we notice that they are not frequent, that they have not occurred consecutively, nor at any definite interval with relation to the administration of insulin. And further, one of the two discordant values usually falls close to a smooth curve joining the neighboring points when the data is represented graphically,

while in no case does the divergent value fall very far away from this line. Therefore, we believe that we are justified in concluding that the value which harmonizes best with the remaining data should be selected as the more probable one, and the other regarded as merely an irregularity of the sort that it is difficult to avoid when a long series of measurements and manipulations must be made in rapid succession. In our opinion it is the comparative regularity of the sugar changes which we have observed in the various portions of the blood rather than the isolated and exceptional variation which is the fundamental part of the whole phenomenon.

According to our experience the sugar curves of those patients which did and those which did not have hypoglycemic reactions are essentially similar as regards relative changes in corpuscles and in plasma. Theoretically it does not appear that any difference on this score should be anticipated. We conclude, therefore, that the study of the corpuscle-plasma distribution of sugar at high levels is not of any aid in predicting whether or not a hypoglycemic reaction is to be expected later.

In the light of our own experience with the direct process for measuring the sugar content of erythrocytes we believe that this method is much more reliable than it has been considered hitherto. In using it the difficulty which we considered would cause the greatest trouble, was the possibility that the plasma might not be removed completely. On account of the usual higher sugar content of the plasma this would have a tendency to lead to values for cell sugar higher than actually exist. However, in only thirty out of 82 measurements did we find the directly determined value to be the higher one, and in only a few of these is the discrepancy great. Therefore, it would appear that this difficulty has been very largely eliminated as a factor in our results. The danger that sugar may be destroyed by glycolysis in the red cells during their separation and manipulation has been considered also. Should this occur it would lead to errors in the opposite direction from that caused by incomplete removal of plasma. It may have been the factor responsible for the fact that several of the directly measured values are obviously too low and out of line with neighboring results. But in a great majority of the instances glycolysis, if it has occurred, has not been of sufficient magnitude

to confuse the general picture. It is possible, of course, that the two tendencies for error—faulty separation of plasma and glycolysis—may have exactly counterbalanced each other in our measurements by the direct method, but it is difficult to believe that this could have happened consistently. If this interpretation is not correct then the computation method as well as the direct method must be condemned in the numerous instances where they have yielded similar values. Therefore, we are of the opinion that when sufficient care is exercised in separation of plasma and preparation of the protein-free filtrate that the directly measured values for erythrocyte sugar are approximately as accurate as those obtained by indirect methods.

The indirect or computation method has given us fairly consistent results in most of our experiments, especially since we have realized the scrupulous care which must be devoted to each measurement which is involved in any way in the calculations. It should be pointed out, however, that this method is particularly insidious because of the slight errors that are quite prone to creep into the measurement of the blood and the preparation of the protein-free filtrates. Thus duplicate analyses of these filtrates may check exactly and the results obtained for whole blood or plasma may not appear to be much out of line with neighboring determinations, yet the deviation from the true value when combined properly with other slight errors may be sufficient to yield totally erroneous results when the erythrocyte sugar is computed. The value 194 obtained by the indirect method in Table III (see data on Blood Sample 2 of Experiment 6) is an outstanding example of the erroneous data which this method can give. This particular value is totally at variance with the remainder of the table, yet if the column containing the direct measurements of erythrocyte sugar were not at hand the discrepancy would not be nearly so obvious. The erratic variations of erythrocyte sugar which have been reported previously are probably to be explained as products of the defects of the indirect method. We do not believe that this method should be employed without some simultaneous measurements by the direct procedure to serve at least as a check and to aid in the elimination of faulty data. What we have found in the case of sugar measurements doubtless applies also when the indirect method alone is used for determination of the other constituents of erythrocytes.

In connection with experiments upon diabetic dogs it will be noted that the sugar content of the corpuscles does not rise to the high levels which we found in human corpuscles. Also the sugar of the plasma declines at a much more rapid rate than that of the erythrocytes. These observations seem to be in line with the view expressed by a number of investigators that the corpuscles of animals are not very permeable to glucose. The paper by Ege (5) gives the references to this literature.

SUMMARY AND CONCLUSIONS.

1. In a series of experiments upon diabetic patients and normal and diabetic dogs no indication has been found that insulin causes a sudden and marked drop of sugar in the erythrocytes preceding that in the plasma or the whole blood.
2. In man the changes take place at about the same rate in all portions of the blood.
3. The direct method of measuring the sugar content of erythrocytes after their separation from plasma gives results as reliable as those obtained indirectly from computations based upon sugar content of whole blood, plasma, and percentage volume of corpuscles.
4. The indirect method is capable of yielding results which may entirely misrepresent the true situation. It should not be used without simultaneous employment of the direct method as a check.
5. The effect of insulin upon the concentration of non-fermentable reducing substances in various portions of the blood is very slight.

We wish to express our obligation to Dr. E. P. Joslin for the interest he has taken in this investigation and for permission to employ some of his patients as subjects. To Dr. Reginald Fitz and Dr. K. D. Blackfan we are also indebted for subjects of our experiments. Mr. Maurice Dionne has assisted us in the manipulations of many of the experiments. Mr. Donald Simons and Mr. Kenneth Burton have aided us in performing the pancreatectomies.

BIBLIOGRAPHY.

1. Foshay, L., *Am. J. Physiol.*, 1925, lxxiii, 470. Foshay, L., and Boyd, D., *Arch. Surg.*, 1927, xv, 397. Foshay, L., *Arch. Int. Med.*, 1927, xl, 661.
2. Rona, P., and Sperling, M., *Biochem. Z.*, 1926, clxxv, 253.
3. Wishart, M. B., *J. Biol. Chem.*, 1920, xliv, 563.
4. Michaelis, L., and Rona, P., *Biochem. Z.*, 1908, xiv, 476.
5. Ege, R., Gottlieb, E., and Rakestraw, N. W., *Am. J. Physiol.*, 1925, lxxii, 76.
6. Folin, O., and Berglund, H., *J. Biol. Chem.*, 1922, li, 213.
7. Hansen, K. M., *Acta med. Scand.*, 1923, lviii, suppl. 4, 172.
8. Macleod, J. J. R., Carbohydrate metabolism and insulin, London and New York, 1926, 187.
9. Wu, H., *J. Biol. Chem.*, 1922, li, 21.
10. Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.
11. Van Allen, C. M., *J. Lab. and Clin. Med.*, 1924, x, 1027.
12. Somogyi, M., *J. Biol. Chem.*, 1927, lxxv, 33.
13. Folin, O., and Svedberg, A., *J. Biol. Chem.*, 1926, lxx, 405.
14. Benedict, S. R., *J. Biol. Chem.*, 1928, lxxvi, 457.

ON THE DETERMINATION OF THE SODIUM CONTENT OF SMALL AMOUNTS OF SERUM OR HEPARINIZED PLASMA BY THE IODOMETRIC METHOD.

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In employing the Kramer and Gittleman (1) iodometric method of sodium analysis of serum, I have found certain modifications of the method necessary. In my hands, as had been reported previously by others, the results obtained by this method were inaccurate and not reproducible. The source of error in the method has been corrected by temperature control at a critical point in the progress of the determination. Furthermore the technique has been improved so that consistent results can be obtained with very small samples.

Kramer and Gittleman recommend that 2 cc. of serum be used for the analysis. It was found necessary to use a very much smaller sample in some of my work; accordingly, the amounts of reagents were decreased proportionately and the same precision was gained for as little as 0.3 cc. of a known sodium chloride solution containing 350 mg. of sodium per 100 cc. as was possible when a 2 cc. sample was used.

However, results varying from 260 to 326 mg. of sodium per 100 cc. of plasma, figures obviously too low, were obtained on analysis of twelve specimens from four normal individuals, regardless of whether 0.3 cc. or 2 cc. of sample were used. Furthermore I was unable to get duplicate results on the same samples of plasma or serum.

Difficulties of this kind were also encountered by Kerr (2) in the determination of the sodium in serum by the Kramer-Gittleman method. Kerr found that the results obtained on analysis of untreated serum (containing albumin and globulin) were often

10 per cent lower than the results obtained on the same samples after removal of the proteins. Kerr found it necessary to remove the proteins before an accurate analysis could be made.

As there was some indication that denaturation and precipitation of proteins occurred on the addition of the alcohol to the solution of serum and potassium pyroantimonate, it was thought that if this precipitation could be avoided the precision of the Kramer-Gittleman method would be greatly improved. For this reason the alcohol was added at 10°, at which temperature the rate of denaturation of the proteins is greatly diminished. (Lepeschkin (3) found the coefficient of reaction velocity of the denaturation of albumin by alcohol to be 110 for a rise in temperature of 10°.) There was no protein precipitate apparent when this procedure was used. The precipitated sodium pyroantimonate was uniformly readily soluble in concentrated hydrochloric acid.

In the presence of the plasma proteins, a precise determination is possible on as small a sample as 0.3 gm. of plasma or serum, when the following method is rigorously employed.

Procedure.

Preparation of Reagents.

Potassium Pyroantimonate Solution.—500 cc. of distilled water are heated to boiling in a Pyrex Erlenmeyer flask. Approximately 5 gm. of potassium pyroantimonate ($K_2H_2Sb_2O_7$) (J. T. Baker) are added with stirring. Boil for from 4 to 6 minutes (not longer). Cool the flask immediately under running water and add 15 cc. of 10 per cent KOH (alcohol-washed). Filter the reagent through double ash-free filter papers into a paraffined bottle. Allow to stand 24 hours before using.[1]

This solution, if allowed to boil too long, becomes reduced. Furthermore, the purity of the samples varies somewhat. It is therefore advisable to measure the oxidizing power of the sample before use. This may be done in the following way.

Put 2 cc. of the solution as prepared above in a 100 cc. Pyrex beaker. Add 15 cc. of distilled water and 5 cc. of concentrated

[1] The reagent is sufficiently stable for 2 months. See Kramer and Gittleman (1) for further precautions before using the reagent.

hydrochloric acid. Then add 2 cc. of the 20 per cent KI solution and titrate with 0.05 N sodium thiosulfate.

Calculation.

$$\frac{\text{Cc. of 0.05 N thiosulfate solution} \times 5 \times 0.575}{2} = \text{mg. of sodium equivalent in}$$

5 cc. of reagent.

The sodium equivalent should not be appreciably less than 4.7 mg. for 5 cc.

20 Per Cent Potassium Iodide.—This reagent should be made up fresh each day.

Starch Solution.—1 per cent starch solution prepared fresh each day.

Potassium Biiodate.—1.6473 gm. of Kahlbaum, Best Grade, potassium biiodate are dissolved in 500 cc. of water. According to Kramer and Gittleman, the solution keeps at least a month.

Sodium Thiosulfate.—12.411 gm. of sodium thiosulfate are dissolved in 1 liter of water. Add a drop of toluene to preserve.

Standardization of Sodium Thiosulfate Solution.—Place 1 cc. of 20 per cent potassium iodide solution in a 100 cc. beaker. Add 0.5 cc. of 3.33 N hydrochloric acid and 10 cc. of distilled water. Then add exactly 2 cc. of the 0.1 N potassium biiodate solution and titrate with the sodium thiosulfate solution from a 5 cc. micro burette, adding the solution quickly until a pale yellow color is reached. Then add approximately 0.5 cc. of a 1 per cent starch solution and titrate slowly to a colorless end-point.

$$\frac{\text{Cc. of 0.1 N biiodate}}{\text{Cc. of sodium thiosulfate}} \times 0.1 = \text{normality of sodium thiosulfate solution.}$$

If the solution is not exactly 0.05 N, the sodium thiosulfate factor found from the following formula

$$\frac{\text{Normality of sodium thiosulfate found}}{0.05} = \text{sodium thiosulfate factor}$$

is used in the calculation as shown below.

Method.

The blood is taken from an arm vein without stasis and the serum or plasma separated by centrifuging. If plasma is to be

used for the determination, 1 mg. of heparin to 5 cc. of blood should be used as anticoagulant. Experiments have shown conclusively that this amount of heparin does not cause an exchange of fluid between the cells and plasma and contains no substances which interfere with the determination.

0.3 to 0.6 cc. (or 0.3 to 0.6 gm. weighed directly in the centrifuge tube when the experiment is made) of serum or heparinized plasma (1 mg. solid heparin to 5 cc. of blood) is placed in a 15 cc. graduated Pyrex centrifuge tube[2] and the volume made up to 1 cc. with distilled water.

5 cc. of the pyroantimonate solution are added and the mixture cooled to 10° (±1°).

Now add drop by drop 1.5 cc. of 95 per cent ethyl alcohol, which has also been cooled to 10°, to the solution from a micro burette with constant, vigorous stirring. This addition of the alcohol should consume at least 30 to 40 seconds.

Remove the tube from the cold water bath; wash off the stirring rod with not more than 1 cc. of distilled water. Put the rod into a 100 cc. Pyrex beaker (in which the titration is to be made later) and stopper the centrifuge tube.

Allow to stand for at least ¾ to 1 hour. Then centrifuge for 5 minutes at about 1500 R.P.M. Remove as much of the supernatant fluid as possible, without disturbing the precipitate, by means of a special pipette, consisting of a test-tube with small glass siphon and suction appliance. It is usually possible to remove all but about 0.2 cc. of the fluid in this manner.

Add 5 cc. of 30 per cent ethyl alcohol, centrifuge, and again remove the supernatant fluid.

Add 5 cc. of concentrated hydrochloric acid (sp. gr. 1.182) and dissolve the precipitate by stirring with the same rod previously used during the precipitation.

Transfer the solution to the same 100 cc. beaker and wash the tube out three times with 5 cc. of distilled water each time.

Fill a 5 cc. micro burette with 0.05 N sodium thiosulfate. Add 2 cc. of freshly prepared 20 per cent potassium iodide solution to the sample and titrate quickly to a pale yellow color. Do not carry the titration till the solution is colorless.

[2] It is advisable to use Pyrex tubes which have been chemically aged.

At this point add approximately 0.5 cc. of a freshly prepared 1 per cent starch solution and titrate to a colorless end-point.

If, instead of the small amounts, from 0.7 to 1.4 gm. of heparinized plasma or serum are to be used for the determination, transfer

TABLE I.

Sodium Content of Ashed and Unashed Plasma.

Sample No.	Unashed.		Ashed.	
	Weight of sample.	Na per 100 gm.	Weight of sample.	Na per 100 gm.
	gm.	*mg.*	*gm.*	*mg.*
1	0.3544	343	0.4643	334
2	0.5910	342	0.5600	341
3	0.4168	331	0.4230	333
4	0.4168	331	0.3835	328
5	0.3560	340	0.9205	331
6	0.3192	339	0.4945	349

TABLE II.

Recovery of Sodium Added to Plasma.

Subject.	Weight of plasma.	Na per 100 gm. Mean of two determinations.	Amount of NaCl solution added.*	Na recovered.
	gm.	*mg.*	*cc.*	*mg. per 100 cc.*
J. K.	0.5008	340	1.00	150
"	0.4823	340	1.00	150
M. M.	0.3150	342	1.00	154
"	0.3320	342	1.00	150
A. P.	0.5075	340	0.50	153
"	0.4278	340	0.50	151
Mouse plasma	0.3870	376	1.00	154
Guinea pig plasma	0.3749	389	1.00	152

* Solution contains 150 mg. of sodium per 100 cc.

this to a 50 cc. Pyrex centrifuge tube and make the volume up to 2 cc. Add 10 cc. of the pyroantimonate solution and 3 cc. of alcohol drop by drop, as above, at 10°. Wash the sample with 10 cc. of 30 per cent ethyl alcohol. Otherwise proceed as above.

Calculation of Sodium in Serum or Plasma.—1 cc. of 0.05 N

thiosulfate solution is equivalent to 1 cc. of 0.05 N iodine solution. 1 equivalent of iodine is liberated by 0.5 of an equivalent of sodium; therefore 1 cc. of 0.05 N thiosulfate solution is equivalent to 1 cc. of 0.025 N sodium, or 0.575 mg. of sodium. Hence

$$\frac{\text{Cc. of thiosulfate solution} \times \text{thiosulfate factor} \times 57.5}{\text{Cc. or gm. of sample}} = \text{mg. of sodium per}$$

100 cc. or 100 gm. of sample.

TABLE III.

Sodium Content of Plasma of Normal Human Beings.

Experiment No.	Subject.	Sample 1. Weight of plasma.	Na per 100 gm.	Sample 2. Weight of plasma.	Na per 100 gm.	Mean, Na per 100 gm.
		gm.	*mg.*	*gm.*	*mg.*	*mg.*
1	A. A. P.	0.4241	339	0.4002	341	340
2	J. K.	0.5057	339	0.4652	341	340
3	O. W.	0.4514	336	0.8117	334	335
4	A. E. M.	0.4283	339			339
5	M. M.	0.4008	340	0.3787	344	342
6	E. S.	0.4000	347	0.4655	347	347
7	E. E. J.	0.3560	340	0.3548*	343*	342
8	A. M. G.	0.3373	340	0.4137	343	342
9	M. B.	0.3549	333	0.3552*	326*	330
10	M. A.	0.3544	343	0.3550*	345*	344
11	G. F.	0.4259	342	0.4960	333	338
12	G. G.	0.5788	343	0.5910	342	343
13	M. D. R.	0.9362	333	0.4658	336	335
14	S. V. G.	0.510	334	0.510	332	333
Average						339
Standard deviation†						±4.7

* Serum.

† The standard deviation is calculated from the formula, standard deviation $\sqrt{\frac{\Sigma d^2}{n-1}}$ where Σd^2 is equal to the sum of the squares of the differences between the individual determinations and the arithmetical mean of the series.

If the cell volume percentage as well as the sodium content, by volume, of the serum or plasma has been determined, the sodium in the whole blood can be calculated.

Experimental Results.

The sodium contents of ashed and unashed human serum and plasma are shown in Table I. These results indicate that the method given above gives accurate results when the determination is made directly on serum or plasma without removal of the proteins, and incidentally that no appreciable amount of sodium remains in combination with the proteins under the conditions at which precipitation of the sodium as pyroantimonate is carried out.

Known amounts of sodium were next added to eight samples of heparinized plasma, the sodium contents of which had been determined as above. That the sodium can be recovered effectively is clearly demonstrated by the results given in Table II.

The results of the determination of the sodium contents of the plasma and serum of fourteen normal human beings (given in Table III) showed variations from 330 to 347 mg. of sodium per 100 cc., with an average mean of 339 and a standard deviation of ± 4.7.

The actual amount of sodium and the uniformity of these results agree with what has generally been found on analysis of the protein-free filtrate or of ashed samples by other investigators.

SUMMARY.

Modifications of the Kramer-Gittleman iodometric method of determination of sodium directly on serum, containing protein, are introduced.

Sodium determinations made by this modified method yield the same results on the ashed and unashed samples.

Sodium which has been added to plasma can be quantitatively recovered.

Duplicate analyses consistently check within 2 per cent and generally within 1 per cent.

The sodium determinations may be made on as little as 0.3 gm. of heparinized (1 mg. of heparin to 5 cc. of blood) plasma or serum.

The sodium content of human whole blood can be calculated when the plasma or serum sodium content by volume is determined and an accurate hematocrit reading made.

The average sodium content of the plasmas of fourteen normal

human beings was 339 mg., standard deviation ±4.7 mg. of sodium per 100 gm. of plasma.

I am grateful to Dr. W. T. Bovie for his encouragement and assistance throughout the progress of this work.

BIBLIOGRAPHY.

1. Kramer, B., and Gittleman, I., An iodometric method for the determination of sodium in small amounts of serum, *J. Biol. Chem.*, 1924-25, lxii, 353.
2. Kerr, S. E., Studies on the inorganic composition of the blood. I. The effect of hemorrhage on the inorganic composition of serum and corpuscles, *J. Biol. Chem.*, 1926, lxvii, 689.
3. Lepeschkin, W., Kolloidchemie des Protoplasmas, Berlin, 1924, 46.

THE CHEMICAL COMPOSITION OF THE ACTIVE PRINCIPLE OF TUBERCULIN.

XI. AN IMPROVED AND SIMPLIFIED METHOD FOR MAKING A STANDARD UNDENATURED TUBERCULIN OF ANY DESIRED STRENGTH AND A METHOD OF CHEMICAL ASSAY.*

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It has been well demonstrated even upon a commercial scale[1] that the tubercle bacillus will grow as abundantly and quickly upon the Long synthetic medium, containing no protein and only known constituents, as upon the old Koch's glycerol broth medium. This, as a starting point, affords us a tuberculin which is suitable for rigid chemical analyses and which, moreover, should yield to a type of chemical standardization otherwise impossible.

It has been concluded in previous publications of experiments in which many different methods of approach were employed, that the active principle in tuberculin which is responsible for eliciting the skin reaction in tuberculous guinea pigs, is a water-soluble protein of the nature of an albumin. Moreover, it is a protein which in its most natural form will crystallize into needles (1) but which through slight manipulation is denatured, becomes less soluble, and gradually loses its biological potency. It appears from previous studies (2) that all of the water-soluble protein in tuberculin is active protein, for no fraction more potent than any other could be separated by fractional precipitation, fractional heat coagulation, or extraction. Only when insoluble protein

* Aided by a grant to Dr. Esmond R. Long from the Medical Research Committee of the National Tuberculosis Association.

[1] In cooperation with the Medical Research Committee, the H. K. Mulford Company and Parke, Davis and Company have grown large quantities of the bacilli on the Long synthetic medium.

fractions are obtained from this originally water-soluble protein, is there any loss in biological activity.

It is obvious, therefore, that a profitable method for manufacturing the most potent tuberculin would be one that secures this protein in concentrated form and with as little chemical manipulation as possible. Koch's original method of preparing O.T. permits of the loss of considerable protein, which is coagulated by heat during evaporation and then filtered off with the bacillary bodies. This coagulable protein has been shown to be some of the active protein (2).

A more recent method (3) (precipitation by $(NH_4)_2SO_4$) has been given a large scale trial by the H. K. Mulford Company. This secures the active protein in dry powder form and with a fairly constant potency (2 mg. is a minimal lethal dose for a 400 to 500 gm. standardized tuberculous guinea pig), but is open to the objection that it is a slow, laborious process permitting considerable denaturation and consequent inactivation of the protein. In a recent report (1) it was calculated that about 50 per cent of the lethal doses were lost in this process during dialysis. Moreover, it was shown that most of the protein prepared in this manner was in the first stage of denaturation or the uncrystallizable stage, for only a very small fraction of the protein can be induced to crystallize. This may be partly due to the drying process.

There is much to be desired, therefore, in the way of a process which will eliminate these objections. Since the active principle which is to be concentrated is a protein, ultrafiltration would seem to be a promising method for concentration without denaturation. In a previous paper on electrodialysis (4) it was noted that the Bronfenbrenner ultrafiltration, electrodialysis apparatus, used exactly as recommended, failed to separate satisfactorily the active principle of tuberculin, although it may be entirely satisfactory for other proteins.

Recently with the suggestion and help of Dr. Julian H. Lewis a method and apparatus were devised suitable for the ultrafiltration of tuberculin in large quantities. A solution of 10 to 12 per cent guncotton in glacial acetic acid was used for impregnating large porous alundum shells. In this apparatus, by simple suction, the non-colloidal constituents of the medium, consisting of crystalloids, other small molecules, and water, can be removed to any desired degree.

Description of Apparatus.

An alundum extraction shell (45 mm. in diameter × 127 mm.) is closed with a 1-hole rubber stopper into which fits a short glass tube to which is attached the suction tube. The shell is then placed in water and considerable water sucked through. While it is still moist it is placed in a 10 or 12 per cent solution of soluble guncotton in glacial acetic acid and the suction continued for 1 minute, at the end of which time the tube is disconnected and the shell

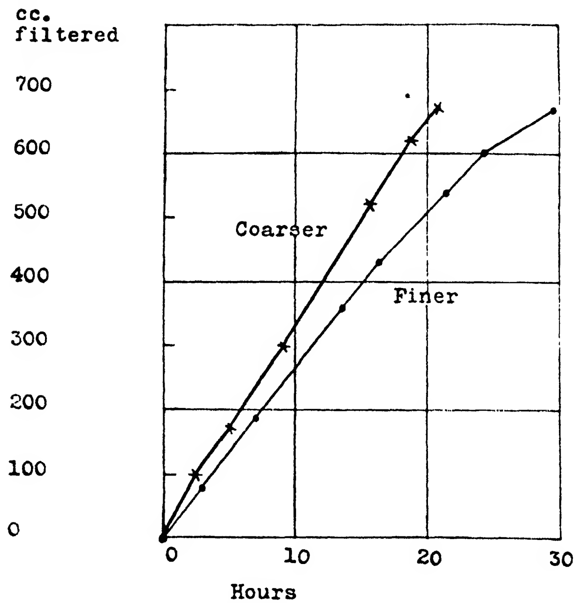

CHART I. Rate of filtration through alundum cups.

removed from the solution and allowed to drain until practically all the excess guncotton has run off. It is then immersed in lukewarm water for several hours and finally washed overnight by filtering water through it. When the filtered water gives no test with Congo red for acid the shell is ready for use. A whole battery of such shells for filtering large quantities of tuberculin can be arranged as indicated in Fig. 1.

Shells of two porosities (dense and medium) were tested and Chart I shows that while the amount of solution passing through the coarser one is greater in the beginning, the rate (about 15 to

20 cc. per hour) in the two cases becomes practically the same, presumably as the membrane adsorbs protein. The same membrane can be used over and over again for days and months provided it is not allowed to dry and is kept covered in water preserved with 1 per cent phenol when not in use. According to the number of such filters hanging in the same lot of tuberculin, a considerable volume of filtrate can be removed in a fairly short time, and the apparatus when once in operation needs scarcely any attention.

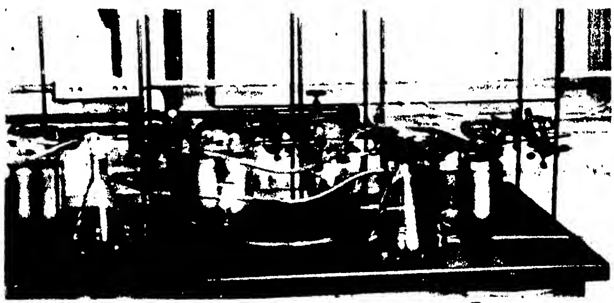

FIG. 1. A, C, and E are batteries of alundum cups impregnated with guncotton and hanging in tanks filled with tuberculin. (A contains two cups; C, four cups; and E, six cups.) One water suction pump, S, evacuates the entire system and water is prevented from backing up into the filtrate flasks by means of a trap, T. The filtrate from the cups in Tank A is collected in flask B and that from the cups in both Tanks C and E is collected in Flask D. Tanks of varying sizes are provided in order to take care of diminishing quantities of tuberculin during the final stages of concentration, and can be modified as the need dictates.

Skin tests on guinea pigs with the two samples of filtrates obtained above showed that none of the active agent filtered through the membranes and gave results as follows:

Cup A.—Coarser alundum (Cenco-porosity RA 360).

Non-filtrable residue.	Tuberculous guinea pig.	5+
	Normal guinea pig.	Negative.
Ultrafiltrate.	Tuberculous guinea pig.	Trace.
	Normal guinea pig.	Negative.

Cup B.—Finer alundum (Cenco-porosity RA 84).

Non-filtrable residue.	Tuberculous guinea pig.	4–5+
	Normal guinea pig.	Negative.
Ultrafiltrate.	Tuberculous guinea pig.	Negative.
	Normal guinea pig.	Negative.

It was found later that if the shells were impregnated with an 8 per cent solution of guncotton or parlodion in glacial acetic acid, the process is greatly speeded up; in fact, as much as tenfold. This, of course, would be an item for consideration in the commercial production of such a product. It was found, for example, that when 10 liters were so filtered in 30 hours, only 8.3 per cent of the protein was lost through the membrane, partly by filtration and partly by adsorption to the membrane.

In the four following experiments the amounts of concentration of the tuberculin were as follows:

Experiment	A.	8.0 times.	2 liters	down	to	250	cc.
"	B.	10.5 "	10 "	"	"	950	"
"	C.	100.0 "	12 "	"	"	120	"
"	D.	50.0 "	13 "	"	"	260	"

The degree of concentration by volume was then checked against the degree of concentration by potency, by means of the lethal test in tuberculous guinea pigs.

This biological test, as well as the skin test, or presumably any biological test, has always been more or less unreliable, due to the fact that we are usually standardizing one unknown (the tuberculin) with the aid of another variable (the guinea pig). The speculations in the literature have been many as to why different healthy guinea pigs receiving the same dose of tubercle bacilli at the same time and kept under identical conditions, react differently at the end of the month. But the fact remains that they do vary and they must therefore be standardized, as best we can. The author's method of standardization is different from the usual Bureau of Standards test (5), but presumably brings the same results. It will therefore be described.

Description of Method for Standardizing Tuberculous Guinea Pigs.

A lot of 400 to 500 gm. guinea pigs is inoculated subcutaneously in the groin, each with 1.0 mg. of a virulent (No. 119 or H37) human strain of bacilli in 1 cc. of an extra fine milky saline suspension. After 1 month they all are tested intracutaneously with a strong tuberculin preparation, used for years and known to give a maximum skin test in a properly sensitive guinea pig. All those animals not giving a maximum reaction are discarded unless they

do react properly at some future time. On occasions, as high as 20 per cent of the guinea pigs do not react properly, which is the percentage of failures allowed by the Bureau of Standards test. After the reaction has subsided—about a week—the guinea pigs are ready for testing an unknown preparation. This rule has been adhered to in all of the skin and lethal tests made in the last 3 years.

Experiments on Concentration of Tuberculins by Ultrafiltration.

As is shown in Table I Tuberculin A was concentrated 8 times by volume and 8 times by potency; Tuberculin B, 10.5 times by volume and 10 times by potency; Tuberculin C, 100 times by volume and only 50 to 60 times by potency; and Tuberculin D, 50 times by volume and 33.3 to 50 times by potency. The discrepancy (in the case of Tuberculin C) is due to the fact already stated that this filtration was made through a membrane of 8 per cent parlodion which allowed some protein to pass. Naturally, the more concentrated the protein solution is, the more protein in proportion is lost. An example of this is Tuberculin C where at the end of the filtration the solution was nearly a 2 per cent protein solution. That the loss was due to the fact stated above was shown by the test in which Filtrate C gave a marked skin reaction (3+, 3 to 4+, 4 to 5+, 5+, 5+) but when some of this filtrate was refiltered through the membrane made of the 10 per cent guncotton solution, the resulting filtrate gave a negative skin reaction.

Tuberculin simply concentrated by the ultrafiltration method as described, will contain the medium constituents in approximately the concentrations that they occur in the original tuberculin. It is possible now to remove all of these salts and small molecules by continually adding water or whatever solution is desired during continued ultrafiltration. I have used a 0.5 per cent phenol solution, so as to protect the protein solution against bacterial contamination during washing. Such a process of purification can be carried on to any extent.

It has been possible by this method, for example, to wash out in a fairly short time the $(NH_4)_2SO_4$ from a tuberculo-protein precipitate so that no trace of clouding occurred with $BaCl_2$. The original concentration of $(NH_4)_2SO_4$ was about 35 per cent and between 5 and 10 volumes of 0.5 per cent phenol were used for

TABLE I.

Tuberculin sample.	Times concentration by volume.	Minimal lethal dose.	Times concentration by potency.	Precipitate per cc. of tuberculin.	Times concentration by volume of precipitate.	N per cc. of tuberculin.	Times concentration by amount of N.	Protein per cc. of tuberculin.	Times concentration by amount of protein.
		cc.		cc.		mg.		mg.	
Filtrate C				0.00023				0.0155	
" " refiltered				0.0000				0.0000	
Tuberculin A		2.00		0.0016		0.0193		0.1200	
" Ac (2000–250 cc.)	8.0	0.25	8.0	0.0101	6.2	0.1160	6.01	0.7250	6.01
" B		1.50		0.0025		0.0246		0.1537	
" Bc (10,000–950 cc.)	10.5	0.15	10.0	0.0250	10.0	0.2632	10.70	1.6450	10.60
" C		0.5–0.75		0.0040		0.0432		0.2700	
" Cc (12,000–120 cc.)	100.0	0.01–0.015	50.0–60.0	0.2400	60.0	2.7640	63.98	17.2750	63.98
" D		0.5		0.0076					
" Dc (13,000–260 cc.)	50.0	0.01–0.015	33.3–50.0	0.2300	30.2				
O. T.		1.50		0.0025					
(Synthetic medium)	10.0	0.20	7.5	0.0070	2.8				

washing. Such a method is quicker and superior to the usual dialysis process, and moreover, concentration of the solution can be effected simultaneously with the dialysis.

The possibility of denaturing the protein during this process of removing all of its natural medium environment has been considered and at the present time it is not possible to make a definite statement upon this point. One experiment, however, would indicate that not much loss in biological potency of the protein follows the washing process. Tuberculin Bc (50 cc.) was washed with 500 cc. of 0.5 per cent phenol and the resulting protein solution was colorless, in contrast to the original tuberculin. The amount of trichloroacetic acid precipitate per cc. of the unwashed tuberculin was 0.02 cc., while after washing it amounted to 0.018 cc. The minimal lethal dose in both cases was the same. Further studies are being made. On the face of it, this method would, however, seem to be the simplest, least drastic, and surest way of freeing protein solutions of anything non-colloidal.

We have, therefore, a method for concentrating and purifying tuberculin to any desired degree, by ultrafiltration, with little or no loss in activity, the loss varying inversely with the time during which the filtration has taken place. In consequence, with this and all the previous evidence that the active principle is a protein, *i.e.* from studies on dialysis and electrodialysis, fractional precipitation, fractional heat coagulation, enzyme and acid hydrolysis, and extensive purification by crystallization of the protein, a chemical assay for tuberculin becomes possible.

The researches mentioned in the previous paragraph indicated that the active principle was a whole protein and not a smaller molecule. It is probably not an impurity adhering to the protein, for purification by recrystallization fourteen times increased instead of decreased the potency (1) and only when the *whole* protein was attacked by proteolytic enzymes (3) was there complete loss in activity. Certainly no satisfactory opposing argument based upon facts has come to the author's attention.

Bieling's (6) claim that he has a product, free from protein, which gives a tuberculin skin reaction, is open to the criticism that the protein tests reported were inadequate. For example, the active product was an alcohol extract and therefore may very possibly have contained glycerol which is always present in

tuberculin. It was shown (7) in 1925 that the biuret test is unreliable in the presence of glycerol. Furthermore, the ninhydrin test, while a very delicate method for detecting amino acids, is not a good test for whole protein, which coagulates and adsorbs the color. No well supported evidence has been submitted to confirm the idea of an adhering active principle.

We may, therefore, proceed upon the idea that the active principle is protein, and moreover, a whole protein. Trichloroacetic acid is a desirable whole protein precipitant and therefore, the amount of precipitate obtained from a standard amount of tuberculin should correspond to the amount of nitrogen (and therefore, protein by calculation) in the solution, and both of these in turn should correspond to the biological potency of the sample. This hypothesis was tested out and the results were found to be surprisingly quantitative.

Method for Chemically Assaying the Tuberculin.

The method consists in taking 5 cc. or a suitable amount of a tuberculin in a Hopkin's vaccine tube and adding to it an equal amount of 20 per cent trichloroacetic acid and letting it stand for several hours. The precipitate is then centrifuged and the amount read (see Fig. 2).

The nitrogen in an equal sample can be determined by making a similar precipitation but this time filter off the precipitate on a small hardened filter paper and wash out the nitrogenous constituents of the medium thoroughly with trichloroacetic acid. The paper containing the precipitate is digested in a Kjeldahl digestion mixture and the nitrogen determined by the micro-nitrogen method as described previously (8). The result is controlled by the amount of nitrogen in a similar filter paper.

Although the concentration as regards volume did not always parallel exactly the concentration by potency, presumably because some of the membranes were not thick enough, the amount of concentration as determined either by potency, or by the amount of trichloroacetic acid precipitate, or the amount of nitrogen, corresponded remarkably well, as is seen in Table I.

By use of the factor 6.25 the amount of protein per cc. in each sample of tuberculin can be calculated from the nitrogen obtained

TABLE II.

Unconcentrated tuberculin.				Concentrated tuberculin.			
Tuberculin sample tested.	Amount injected.	Protein in dose.	Reaction.	Tuberculin sample tested.	Amount injected.	Protein in dose.	Reaction.
	cc.	*mg.*			*cc.*	*mg.*	
A (less than 6 wks. growth).	3.0	0.36	Death, 24 hrs.	Ac (concentrated 8 times).	1.00	0.72	Death, 24 hrs.
	2.0	0.24	" 24 "		0.50	0.36	" 24 "
	1.5	0.18	Lived.		0.25	0.18	" 24 "
	1.5	0.18	"		0.10	0.07	Lived.
	1.0	0.12	" (Died in 5 days.)				
B (6 wks. growth).	2.0	0.30	Death, 24 hrs.	Bc (concentrated 10.5 times).	0.20	0.328	Death, 24 hrs.
	1.5	0.23	Lived.		0.15	0.246	" 24 "
	1.5	0.23	Death, 12 hrs.		0.15	0.246	Lived.
	1.2	0.18	" 70 "		0.11	0.180	"
	1.0	0.15	Lived. (Died in 5 days.)		0.10	0.164	" (Died in 4 days.)
					0.10	0.164	"
C (8 wks. growth).	1.25	0.341	Death, 12 hrs.	Cc (concentrated 100 times).	0.03	0.519	Lived (?).
	1.00	0.273	" 12 "		0.03	0.519	" (?).
	0.75	0.204	" 24 "		0.02	0.346	" (?).
	0.50	0.136	" 24 "		0.02	0.346	Death, 24 hrs.
	0.50	0.136	Lived.		0.02	0.346	" 24 "
	0.36	0.100	"		0.015	0.259	" 24 "
					0.015	0.259	Lived.
					0.015	0.259	"
					0.01	0.173	Death, 12 hrs.
					0.01	0.173	Lived.
					0.01	0.173	"

D (12 wks. growth).	1.00	0.500	Death, 48 hrs.	Dc (concentrated 50 times).	0.015	0.232	Death, 16 hrs.
	0.75	0.375	" 20 "		0.015	0.232	Lived.
	0.50	0.250	" 60 "		0.01	0.155	Death, 18 hrs.
	0.50	0.250	" 28.5 "				
	0.35	0.180	Lived.				
	0.20	0.100	"				

and then by comparing this figure with the amount of precipitate obtained per cc. of each tuberculin; it has been shown that about 0.0674 gm. of protein = 1 cc. of precipitate. It is simple, therefore, to determine the amount of trichloroacetic acid protein precipitate per cc. of any sample of tuberculin and by use of the factor 0.0674 to calculate the amount of protein present.

In this way, the amount of protein in each dose administered was calculated and Table II shows that the minimal lethal dose seems to be a fairly constant amount of protein (approximately 0.2 mg.) and this is independent of the volume in which it is administered. For example, as little as 0.01 cc. of the most potent and as much as 2.0 cc. of the least potent sample were required to kill a tuberculous guinea pig.

In previous studies (2) it was shown that 2 mg. of the dry powder (of which 78 per cent or 1.56 mg. was whole protein) made by the $(NH_4)_2SO_4$ process, were a minimal lethal dose for a tuberculous guinea pig. Therefore, the protein as obtained by simple ultrafiltration is roughly 8 to 10 times per unit as potent as is the protein after it has been subjected to chemical manipulation and drying. The protein is, therefore, less denatured biologically. Moreover, it is also less denatured chemically, for it has just been found that roughly as many crystals can be secured from an amount of tuberculin concentrated by this method (equivalent to 1 liter of original Tuberculin C from a 6 weeks growth) as from an amount of the dry powder made by the $(NH_4)_2SO_4$ process, which was obtained from 40 to 50 liters of original tuberculin. It is hoped that in the future sufficient crystals can be obtained by means of this method for quantitative analyses. While the idea has always been voiced and all text-books state that crude raw tuberculin is more potent than any of the purified products obtained from it, no such quantitative comparison has been available.

Not only is the tuberculin obtained by this ultrafiltration method 8 to 10 times as potent for tuberculous guinea pigs as the protein obtained in powder form, but also it is about 10 times as toxic for perfectly normal uninfected animals. For example, it has been demonstrated frequently that 100 to 150 mg. of the powdered protein obtained by the $(NH_4)_2SO_4$ method will kill a normal guinea pig within 48 hours. Of tuberculin concentrated by the ultrafiltration method 0.8 to 1.0 cc. (containing 12.6 to 17.3 mg.

of protein) kills normal guinea pigs within 24 hours. This increased toxicity is not due to a concentration of the medium constituents as may be partly true in the case of O.T.

In the case of both preparations, therefore, it requires 50 to 100 times as much to kill a normal guinea pig as to kill a tuberculous animal. In other words, the difference is a quantitative one and it cannot be said that the tuberculin reaction is truly specific but merely quantitatively specific. Moreover, less striking than these large quantitative differences between normal and tuberculous animals are the smaller quantitative differences in reacting power noted between individual tuberculous animals. In Table II it is evident how difficult it is to say, for example, just exactly what is the minimal lethal dose of most of the preparations. In many cases when the border line of dosage is reached some guinea pigs will die within 48 hours, while others will survive for varying lengths of time or even not die at all. This is true, even in these experiments, where all of the guinea pigs were standardized previously by means of the skin test. A statement, therefore, of the minimal lethal dose can, at best, be but an average or a matter of judgment.

At the bottom of Fig. 2 are shown the amounts of precipitate in two O.T. preparations of tuberculin made by the original Koch method. To the left is the amount (0.035 cc.) of trichloroacetic acid precipitate in 5 cc. of an O.T. tuberculin made from a 6 weeks culture on the Long synthetic medium, boiled for an hour, filtered through the Berkefeld candle, evaporated to 0.1 of the original volume, refiltered through paper, and preserved with 0.5 per cent phenol. This precipitate is to be compared with Tuberculin Bc in the row above (0.125 cc.) which represents the precipitate from a corresponding culture, but concentrated by ultrafiltration. Obviously, much whole protein is lost by the usual method of preparing O.T. tuberculin.

The potency of the O.T. (synthetic) tuberculin, however, was not correspondingly reduced, 0.2 cc. (0.096 mg. of protein) being a minimal lethal dose, whereas of the Tuberculin Bc, which was a corresponding culture, but concentrated by ultrafiltration, 0.15 cc. (0.246 mg. of protein) was required to produce death. The apparent increased toxicity is being further investigated. It is true that a similarly concentrated but uninoculated broth does cause considerable discomfort and apparent shock even in normal animals.

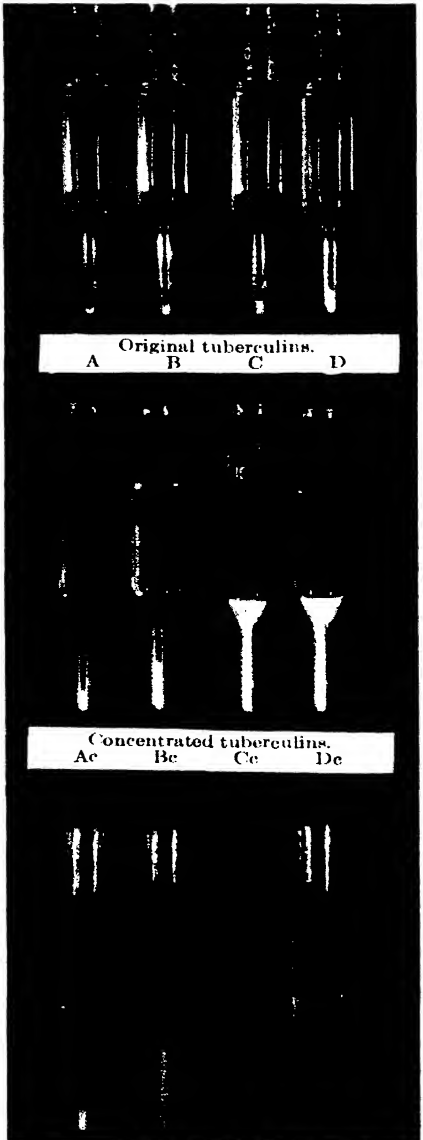

FIG. 2. Chemical standardization of tuberculin.

The second tube in the bottom row (Fig. 2) shows the amount of precipitate (about 0.75 cc.) in 5 cc. of regular commercial O.T. (Parke, Davis and Company). Much of the precipitate here is due to substances in the peptone medium itself. The exact amount of such extraneous precipitate cannot be definitely stated, since a control determination similarly made upon 5 cc. of concentrated but uninoculated peptone broth medium gave a sticky gummy precipitate which could not be centrifuged off successfully.

From these observations on the varying potency of different preparations made by different methods it is therefore evident that the method described in this paper for assaying tuberculin chemically cannot be applied to tuberculins made upon protein-containing media, nor to tuberculin protein purified by almost any

TABLE III.

Tuberculin sample.	Age of culture.	Tuberculin per bottle, average.	Protein per bottle.	Amount of trichloroacetic acid precipitate per cc. tuberculin.	Protein per liter tuberculin.	Minimal lethal dose.	Protein in minimal lethal dose.
	wks.	*cc.*	*gm.*	*cc.*	*gm.*	*cc.*	*mg.*
A	5–6	150.0	0.0161	0.0016	0.1078	2.0	0.24
B	6–7	142.0	0.0239	0.0025	0.1685	1.5	0.23
C	8	132.7	0.0357	0.0040	0.2696	0.5–0.75	0.136–0.204
D	12	112.3	0.0576	0.0076	0.5122	0.5	0.250

chemical process, for there may be stages in the denaturation of the protein where biological activity is lost before a decrease in precipitability by trichloroacetic acid is evident.

The method is entirely satisfactory, however, if used exactly as described; *i.e.*, following concentration of the tuberculin made on a synthetic medium by means of ultrafiltration without any chemical manipulation of the protein.

By means of this chemical standardization of tuberculin an important fact was easily disclosed and consequently the statements in the literature were quantitatively confirmed; namely, that the older the culture growth, the more potent the tuberculin. Fig. 2 and Table III show that this is true and conversely, therefore, that the potency of any tuberculin made upon a non-protein synthetic medium may be determined by measuring the precipitate

obtained from a specified amount of tuberculin. Much more protein per liter is found as the age of the culture increases. Tuberculin D, for example, was a 12 weeks culture and contained as much protein per cc. as Tuberculin Ac which was a 5 to 6 weeks culture concentrated 8 times by volume and 6 times by protein content. While some of this increase is due to simple evaporation in the incubator, much of it is not, for in the older cultures, more protein is found per bottle, as noted in Table III. This result confirms some work I did with R. D. Coghill in 1927 at Yale University (unpublished results) where with increased age of the culture more dry protein made by the $(NH_4)_2SO_4$ process was found in the culture medium and correspondingly less in the bacilli.

The following conclusion therefore may be drawn: A long period of incubation of the tubercle bacillus culture (3 months) would prove a profitable method for manufacturing tuberculin, at the end of which time, considerable evaporation has automatically taken place and the active principle is naturally extracted from the bacilli, saving the labor of extraction in the laboratory. Moreover, much less denaturation has taken place by this natural extraction, than by methods usually used for extracting the bacilli.

SUMMARY.

An improved method is outlined for separating out the active principle of tuberculin. The essential features are growth of tubercle bacilli upon a liquid non-protein synthetic medium, and ultrafiltration of the bacteria-free Berkefeld filtrate. The advantages over all previous methods are: (1) A tuberculin of any desired potency is produced without loss of the active principle. (2) An undenatured product is produced, as evidenced by the fact that its potency (about 0.2 mg. of protein is a minimal lethal dose for a 400 to 500 gm. tuberculous guinea pig) is roughly 10 times the potency per unit of the most potent dry powder preparation previously obtained (2 mg. = minimal lethal dose). Moreover, a considerable portion of this protein will crystallize. (3) Even in the crude form the tuberculin after concentration by ultrafiltration is fairly free of concentrated irritating constituents and with very little more work it can be washed practically free of all of these substances.

A chemical assay for tuberculin made upon a non-protein medium is submitted and has proved to be consistent with the biological assay (lethal test). It is based upon extensive evidence submitted in previous publications that the active principle of tuberculin is a whole protein, and that all of the water-soluble protein is active protein. Trichloroacetic acid precipitates this protein active principle quantitatively. In the filtrates which contained a small amount of active principle, as evidenced by the skin test in tuberculous guinea pigs, there was also a small amount of trichloroacetic acid precipitate formed upon standing, and conversely where there was no biological activity, there also no precipitate formed.

After concentration of four different tuberculin preparations by ultrafiltration, a nearly quantitative agreement occurred in the determination of concentration made by the three different methods: amount of trichloroacetic acid precipitate, amount of nitrogen per unit of volume, and the minimal lethal dose for tuberculous guinea pigs.

It was found that the potency of any tuberculin preparation increases with the age of the culture up to 3 months incubation at least. The effect of longer periods of incubation is being studied.

The tuberculin reaction, as indicated by the lethal test, is not truly specific but only quantitatively specific, since 50 to 100 times the dose for a tuberculous guinea pig produces a corresponding reaction in a normal, unsensitized animal.

A simple method for preparing and standardizing tuberculin, therefore, consists of the following steps.

1. Plant a virulent human stain of tubercle bacilli upon the Long non-protein synthetic medium and allow it to incubate 3 months.

2. Remove the bacilli completely by filtration through the Berkefeld candle and concentrate the tuberculin to any desired degree by filtering it, preserved with 0.5 per cent phenol, through alundum extraction shells previously impregnated with a 10 to 12 per cent solution of soluble guncotton in glacial acetic acid and washed free of the acid. (The rate is 15 to 20 cc. per hour through a cup 45 mm. in diameter $\times$ 127 mm.) If speed is desirable and quantitative recovery is not essential, the process may be hastened tenfold by using membranes made of an 8 per cent solution of

soluble guncotton or parlodion in glacial acetic acid. Concentration by this method can be carried on to any desired degree, and further purification can be effected by washing the protein by continued ultrafiltration.

3. Standardize the potency of the tuberculin by determining the amount of precipitate obtained when trichloroacetic acid is added to a standard amount of the tuberculin. Obtain the amount of protein and the minimal lethal dose by calculation (1 cc. of the precipitate corresponds to 0.0674 gm. of protein and about 0.2 mg. of this protein is a minimal lethal dose for a 400 to 500 gm. tuberculous guinea pig).

I wish to express my appreciation to Dr. E. R. Long through whom this research has been made possible and whose encouragement and interest have been helpful. I also wish to acknowledge the careful assistance of Miss Nelle Morley who prepared the tuberculin used in these studies.

BIBLIOGRAPHY.

1. Seibert, F. B., *Am. Rev. Tuberc.*, 1928, xvii, 402.
2. Seibert, F. B., *Am. Rev. Tuberc.*, 1928, xvii, 394.
3. Long, E. R., and Seibert, F. B., *Am. Rev. Tuberc.*, 1926, xiii, 393, 398, 404, 408, 431, 441, 448.
4. Seibert, F. B., and Hanke, M. T., *J. Biol. Chem.*, 1928, lxxvi, 535.
5. Schroeder, E. C., and Brett, G. W., *J. Am. Vet. Med. Assn.*, 1919, liv, 357.
6. Bieling, R., *Centr. Bakt.*, *1. Abt.*, *Orig.*, 1924, xliii, 56.
7. Seibert, F. B., and Long, E. R., *J. Biol. Chem.*, 1925, lxiv, 229.
8. Seibert, F. B., *J. Biol. Chem.*, 1926, lxx, 265.

THE α AND β FORMS OF DIACETONE METHYLMANNOSIDE.

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Some time ago we prepared diacetone methylmannosides,[1] on the one hand, by acetonylization of the normal α-methyl-*d*-mannoside, on the other, by methylation of diacetonemannose. The first method led to a dextrorotatory diacetonemannoside. Diacetonemannose, on methylation by Freudenberg's method with metallic sodium and methyl iodide, formed a levorotatory diacetone methylmannoside; on methylation by silver oxide and methyl iodide by Purdie's method a dextrorotatory diacetonemannoside was obtained. We then assumed that the two dextrorotatory forms were identical α-isomers and the levorotatory their β-isomer.

At the time that work was done the lactal structures of the normal mannosides were not yet known, nor was the lactal structure of diacetonemannose known. Since then Levene and Meyer[2] have found that the normal methylmannoside has the $<1,5>$ lactal structure and recently Goodyear and Haworth[3] have shown that diacetonemannose has the $<1,4>$ lactal structure. *In view of the new information the question arose as to whether the three substances were of the same lactal structure.* The previously prepared diacetone methylmannosides differed not only in the directions of their optical rotations, but the two dextrorotatory forms differed considerably in the numerical values of their rotations. By a method of purification which is described in the experimental part, it was possible to raise the numerical values of the rotations of both dextrorotatory substances, but that of the one

[1] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1924, lix, 145.

[2] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1924, lx, 167.

[3] Goodyear, E. H., and Haworth, W. N., *J. Chem. Soc.*, 1927, cxvi, 3136.

derived from diacetonemannose was raised considerably more than the other, so that now the highest dextrorotation of the substance obtained from the normal mannoside is $[\alpha]_D^{20} = +56°$ and that of the other is $[\alpha]_D^{20} = +46.6°$. With a larger supply of crude material on hand, the rotation of the second substance undoubtedly could be raised high enough to equal that of the first. The nature of the lactal structure was tested by the rate of hydrolysis of the various forms. Irvine and Burt[4] have found that the normal methylmannoside hydrolyzes at a much slower rate than the γ form, as seen from Table I.

TABLE I.

Time.	α-Methylmannoside. $[\alpha]_D$	γ-Methylmannoside. $[\alpha]_D$
min.	*degrees*	*degrees*
0	+79.4	+79.4
30	78.6	48.7
90	76.3	15.1
120	75.6	Constant.

We now find that all three preparations of diacetone methylmannoside hydrolyze at the same rate and that all of them are completely hydrolyzed within 90 minutes. Hence it is warranted to assume that they all possess the $<1,4>$ lactal structure. If this is correct, then *the conclusion follows that acetonylization changes the $<1,5>$ lactal structure of methylmannoside into the $<1,4>$ lactal structure.* In harmony with this assumption is the fact that the acetonylization of the normal methylmannoside proceeds very slowly, so that the acetonylization of 40.0 gm. is accomplished in 48 hours, whereas acetonylization of the same quantity of the γ form is accomplished in 1 hour.

This observation emphasizes the importance of the caution which it is necessary to exercise in formulating the lactal structure of a given sugar on the basis of the structure of its derivatives.

In Table II are given the sum and the difference of the molecular rotations of the α and β forms of the diacetone methylmannoside. In harmony with other $<1,4>$ lactal derivatives, the numerical value of the sum of the rotations of the α and β forms is very low.

[4] Irvine, J. C., and Burt, W., *J. Chem. Soc.*, 1924, cxxv, 1343.

TABLE II.

	$[\alpha]_D^{20}$	$[M]_D^{20}$	Σ	Δ
	degrees	*degrees*		
α form	+56.0	+15,120	4050	26,190
β "	−41.0	−11,070		

EXPERIMENTAL.

Diacetone Methylmannoside from Diacetonemannose.—The dextro form of diacetone methylmannoside was prepared from diacetonemannose by methylation with silver oxide and methyl iodide. The product had a rotation of $[\alpha]_D^{20} = +24.6°$. Several portions of about 50 gm. of this syrup were dissolved in petrolic ether (b. p. 40–60°) and cooled intensely with a mixture of solid CO_2 and alcohol. A thick syrup settled out and the clear supernatant liquid was decanted. The petrolic ether solution was concentrated to a thick syrup which had a rotation of $[\alpha]_D^{20} = +26.5°$. It was redissolved in petrolic ether and the cooling process repeated. This time the remaining syrup had a rotation of $[\alpha]_D^{20} = +36°$.

In this manner a total of 20 gm. of syrup having this rotation was obtained. This was again fractionated out of petrolic ether and on distillation of the product a syrup was obtained which had a rotation of $[\alpha]_D^{20} = \frac{+1.64° \times 100}{1 \times 3.52} = +46.6°$. This analyzed as follows for methoxyl:

0.1248 gm. substance: 0.1014 gm. AgI.
$C_{13}H_{22}O_6$. Calculated, OCH_3 11.30. Found, OCH_3 10.72.

Levo Form.—Diacetonemannose on treatment by Freudenberg's method yielded a product with the specific rotation of $[\alpha]_D^{20} = -41°$. On treatment by the procedure employed for purification of the dextrorotatory form the value of the optical rotation remained unchanged.

Acetonylization of α-Methylmannoside.—The preparation of this product was repeated as previously described. 100 gm. of α-mannoside were shaken with 2000 cc. of acetone containing 1 per cent of hydrochloric acid and the acetone-hydrochloric acid renewed each day until all of the sugar was dissolved. This operation required about 5 days. The extracts were combined and

treated by the procedure previously described. The syrup which was obtained in this manner was further fractionated by extraction with petrolic ether (40–60°). Adhering impurities were in this way removed. The final syrup distilled mainly at 105°, p = 0.2 mm., and had the following rotation in methyl alcohol.

$$[\alpha]_D^{20} = \frac{+2.26^\circ \times 100}{1 \times 4.04} = +56.0^\circ.$$

0.1998 gm. substance: 0.1650 gm. AgI.
$C_{13}H_{22}O_6$. Calculated, OCH_3 11.3. Found, OCH_3 10.9.

Diacetone Methylmannoside from γ-Methylmannoside.—γ-Methylmannoside was prepared according to the directions of Irvine and Burt.[4] The syrup which was obtained after removal of the ethyl acetate was redissolved in this reagent and filtered from any insoluble material. This filtrate was concentrated under diminished pressure at room temperature. The remaining traces of ethyl acetate were removed by repeated distillations with dry acetone. A dry crumbly mass (about 40 gm. in weight) then remained. 2 liters of cold dry acetone containing 1 per cent hydrochloric acid were added and the suspension was shaken at 10°. In about 1 hour all of the sugar had gone into solution. Shaking was, however, continued for another hour. The acid was neutralized with sodium methylate and the acetone removed under diminished pressure. The syrup which remained was taken up in dry ether, filtered from the sodium chloride, and again concentrated. The product distilled at 105°, p = 0.2 mm., and had a rotation of

$$[\alpha]_D^{20} = \frac{+2.26^\circ \times 100}{1 \times 4.04} = +56.0^\circ.$$

It analyzed as follows.

4.510 mg. substance: 9.490 mg. CO_2 and 3.380 mg. H_2O.
0.1346 gm. " : 0.1116 gm. AgI.

$C_{13}H_{22}O_6$.	Calculated.	C 56.9,	H 8.08,	OCH_3 11.31.
	Found.	" 57.3,	" 8.20,	" 10.93.

Hydrolysis of the Several Diacetone Methylmannosides.—0.250 gm. of each mannoside was suspended in sealed tubes in 5 cc. of 0.1 N

aqueous hydrogen chloride and the tubes were placed in a boiling water bath. The tubes were opened after 90 minutes and the optical rotations measured. The results are given in Table III.

TABLE III.

	A	B	C	D	E
	degrees	*degrees*	*degrees*	*degrees*	*degrees*
Initial........................	+46.7	−41.0	+56.0	+56.0	+79.4
After 90 min. (calculated as methyl mannoside).........	+16.7	+16.7	+16.7	+16.7	+73.5

A, methylation of diacetonemannose by Ag_2O + MeI.
B, methylation of diacetonemannose by Na + MeI.
C, from α-methylmannoside.
D, from γ-methylmannoside.
E, hydrolysis of α-methylmannoside.

The final rotation of all solutions except for the α-methylmannoside was $\alpha = +0.55°$ ($l = 1$ dm.). The concentration calculated as methylmannoside was c = 3.24.

STUDIES ON THE OXIDATION OF LUCIFERIN WITHOUT LUCIFERASE AND THE MECHANISM OF BIOLUMINESCENCE.

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In five groups of luminous animals it can be shown that luminescence is connected with the oxidation of a substance, luciferin, in aqueous solution, in presence of an enzyme, luciferase, and dissolved oxygen. In the mollusc, *Pholas dactylus*, Dubois (1) has been able to oxidize *Pholas* luciferin with luminescence by $KMnO_4$, H_2O, BaO_2, PbO_2, and hypochlorites. I have been unable to oxidize *with luminescence* the aqueous solutions of luciferin from an ostracod crustacean, *Cypridina hilgendorfii*, by the above oxidizing agents and others to be described below. Oxidation of *Cypridina* luciferin takes place but no light appears. The oxidation product (oxyluciferin) formed can be reduced to luciferin again, whether luciferase is present or not, by nascent hydrogen, sodium hydrosulfite, and other reducing agents (2, 3).

I have already (1918) given evidence to indicate that the reaction is of the following type:

$$2 \text{ luciferin } (LH_2) + O_2 = 2 \text{ oxyluciferin } (L) + 2H_2O$$

similar to the autooxidation of a leuco-dye, rather than the autooxidation of a substance like benzaldehyde, by peroxide formation. We may, as further evidence against the view that luciferin oxidizes with peroxide formation, state that diphenylamine, a negative catalyst for the oxidation of benzaldehyde, has no effect on the luminescence of an oxidizing mixture of luciferin and luciferase. As oxyluciferin is readily reduced by nascent hydrogen at cathodes (4) one might suppose that luminescence of luciferin could be observed at anodes, where nascent oxygen is formed. The following combinations of electrolytes and electrodes all gave negative results with potentials varied from 0 to 6 volts: (1)

luciferin in Na_2SO_4 solution, anode and cathode of Mn, Al, Ag, Ni, Sn, Cu, Fe, Pb, Zn, Cd, Pt; (2) luciferin in sea water, same electrodes as above; (3) luciferin + $CaCO_3$ powder (to neutralize acid) in Na_2SO_4 solution, Pt cathode and anodes of platinized Pt, palladinized Pt, and palladinized Au; (4) luciferin in NaBr, NaCl, and NaI solution, Pt electrodes; (5) luciferin in $NaHCO_3$ solution, Pt electrodes; (6) luciferin in 0.5 M phosphate buffer, pH = 7.3, Pt electrodes.

Oxidation by colloidal solutions of metals and metal hydroxides all gave negative results also. These included Pt, Rh, Ir, Ru, V, Au, Ag, Ni, Fe, Co, and Cu, mostly prepared by the Bredig arc or by reduction methods. Luciferin with colloidal Pt or Pd through which pure oxygen is passed gives no light, nor do platinized or palladinized surfaces saturated with oxygen and placed in luciferin solution, although the same surfaces saturated with hydrogen readily reduce oxyluciferin. Platinized or palladinized surfaces in air or oxygen do oxidize luciferin readily but no luminescence appears.

There is also no luminescence when we add directly to luciferin small amounts of various oxidizing agents such as $KMnO_4$, $K_2Cr_2O_7$, CrO_3, H_2O_2, Na_2O_2, BaO_2, MnO_2, PbO_2, V_2O_5, titanium oxide (white), ozone, benzoyl peroxide, disuccinyl peroxide, Ag_2O, HgO, $FeCl_3$, ferricyanides, chlorates, perchlorates, persulfates, perborates, Mn or Cu salts, active catalytic (for $2\,CO + O_2 = 2CO_2$) Fe_2O_3, Cl_2, Br_2, or I_2, hypochlorites, hypobromites, or hypoiodites. Again I must emphasize the fact that, although many of these substances oxidize luciferin, no luminescence appears. The oxidation-reduction potential of the luciferin-oxyluciferin system has been discussed by me in a recent paper (3).

Insulin is also without action on luciferin or luciferase, or the luminescence resulting from a mixture of the two.

Finally, luciferin cannot be oxidized with luminescence by oxidases, peroxidases, or various plant and animal extracts. Indeed, the luciferase of other luminous species will not oxidize *Cypridina* luciferin with luminescence unless the luminous animal is very closely related (5).

Some biological substances, like peptone, will luminesce with strong alkali and bromine water but crude luciferin solution gives no more luminescence under these circumstances than does an

extract of *Cypridina* containing only oxyluciferin. It is not luciferin but some other organic material in the luciferin solution which luminesces. This sort of luminescence can have no significance for the subject under discussion.

Only when luciferase is added directly to luciferin solution does luminescence occur, but even then there is no interchange of charges between luciferin and luciferase such as occurs in the oxidation of iodides by bromine water. This can be shown by a simple procedure similar to that of Ostwald's "action at a distance" experiment. Luciferin in Na_2SO_4 solution is placed in one vessel connected with a second vessel by a salt bridge and Pt electrodes. In the second vessel is placed luciferase in Na_2SO_4 solution. No luminescence at the electrode in luciferase or in luciferin ever appears. Light emission must be an indirect result of the oxidation of luciferin, otherwise some luminescence should appear in this experiment.

All these negative results lead to the conclusion that luciferin is not the source of the luminescence but merely an accessory, and that luciferase *is* the body emitting light, a view I expressed (6), after study of the color of the luminescence resulting from a mixture of luciferin and luciferase prepared from different animals. Luciferin from a firefly whose light is reddish mixed with luciferase from a firefly whose light is yellow results in a yellow luminescence and *vice versa*. The color of the light from such mixtures is therefore determined by the animal supplying the luciferase (7).

It would seem that luciferase must be excited to luminesce by energy supplied by the oxidation of luciferin, in line with the view of Kautsky and Zocher (8) on luminescence in silicon compounds.

According to this view, luminescence is due to excited or energy-rich molecules returning to the normal state, just as we know luminescence of atoms is due to the change from the excited (energy-rich) to the normal state. Siloxene is a silicon compound whose oxidation by $KMnO_4$, H_2O_2, *etc.* results in a reddish luminescence. The energy from the oxidation of some siloxene molecules is transferred to others, converting them to energy-rich molecules which luminesce on return to the normal state. Siloxene on exposure to ultra-violet light will also exhibit reddish luminescence (fluorescence). In this case the energy of the ultra-violet radiation goes to form energy-rich molecules.

Siloxene oxidizes in several steps, finally forming silicic acid. If it is so completely oxidized that its own luminescence is no longer excited, and rhodamine B adsorbed on the oxidized siloxene, treatment of the material with acid permanganate will result in a yellow luminescence characteristic of the yellow fluorescence spectrum of rhodamine B. The oxidized siloxene molecules have transferred some of their energy to rhodamine B, resulting in luminescence.

Can oxidized siloxene transfer some of its energy to luciferase (or luciferin), causing luminescence in these substances? To test this possibility I have mixed well oxidized siloxene hydroxide[1] with luciferase (and also with luciferin) and then added acid permanganate solution to the mixture, but no luminescence resulted in either case. As this siloxene hydroxide added to rhodamine B gave good luminescence on mixing with acid permanganate,[2] I conclude that neither luciferin nor luciferase molecules can be excited to luminesce in this way. Luciferin or luciferase adsorbed on Patrick's silicic acid jel or permutit also give no luminescence when mixed with acid permanganate. It is rather disappointing to find that siloxene is unable to transfer its energy of oxidation to luciferase and cause this substance to luminesce.

Thinking that fluorescent[3] dyes might be excited to luminesce in the presence of luciferin undergoing oxidation, I have mixed luciferin with rhodamine B, fluorescein, quinine sulfate, and eosin and shaken in air. No luminescence appeared and there was also no luminescence on adding acid permanganate. The above fluorescent dyes also show no luminescence when mixed with luciferase solution.

[1] The material was kindly prepared for me as the bromide by Dr. S. O. Miller and Mr. J. R. Bates of the Chemistry Department. When water is added to the bromide, siloxene OH is formed. It is then treated with successive small amounts of acid permanganate till no more luminescence appears.

[2] Acid permanganate will give a faint luminescence on being mixed with aqueous rhodamine B, but if the rhodamine B is first mixed with oxidized siloxene OH, a bright yellow luminescence occurs when acid permanganate is added.

[3] The fact that these dyes fluoresce is an indication that their molecules are easily excited by radiation and *might* be excited by the energy of chemical reactions.

We may inquire finally whether luciferase can be excited to luminesce (fluoresce) by radiation of any kind. While it is true that the luminous organs of some forms fluoresce in near ultra-violet light ($\lambda = 0.40$ to $\lambda = 0.30$) (9), *Cypridina* material shows no fluorescence on exposure to near ultra-violet,[4] x-ray, or cathode rays.[5]

The only condition under which I have ever noted any luminescence of luciferin without luciferase is in non-aqueous solvents. If dry powered *Cypridinæ* are extracted with 95 to 99 per cent alcohol (in which luciferin but not luciferase is soluble), filtered through several layers of filter paper, and this alcoholic luciferin solution treated with ozonized turpentine or minute amounts of solid $KMnO_4$, disuccinyl peroxide, $K_3Fe(CN)_6$, $PtCl_4$, $PdCl_2$, and Ca hypochlorite, a faint luminescence will result. The alcohol alone gives no luminescence with those reagents and an alcohol extract of dried *Cypridinæ* whose luciferin had been oxidized also gave no luminescence with $KMnO_4$. Toluene and acetone extracts of dried *Cypridinæ* give no luminescence with $KMnO_4$. Luciferase is insoluble in both solvents; luciferin is soluble in acetone but not in toluene.

Many oxidizing agents, like solid quinone, MnO_2, PbO_2, Na perborate, BaO_2, $KClO_4$, $FeCl_3$, iodine, and bromine gave no luminescence with the alcoholic extract. Finally, it should be noted that the alcoholic luciferin extract will luminesce faintly if merely heated to about 70°, while the addition of oxidizing agents gives a much brighter luminescence. Aqueous solutions of luciferin, even if luciferase is also present, will not luminesce at this temperature. It is possible that in alcohol the oxidation proceeds at the surface of luciferase particles, which pass the filter paper, suspended in the liquid. The luminescence is not to be compared in brightness with that in aqueous solutions with luciferase and I mention these results merely as a matter of record, as the only cases where *Cypridina* luciferin alone will luminesce. I do not

[4] Exposure of *Cypridina* powder to ultra-violet light at liquid air temperature results in no pronounced fluorescence.

[5] A test kindly made for me by Dr. W. D. Coolidge of the General Electric Company. Cathode rays outside the tube from 1 milliampere at 200,000 volts for 10 seconds caused no luminescence of dried *Cypridina* powder or of luciferase or luciferin solution.

believe they are necessarily significant for the theory of bioluminescence because so many organic substances luminesce on oxidation with *strong* oxidizing agents.

To say that luminescence intensity depends on reaction velocity of oxidation of luciferin is not sufficient. Luciferin can be oxidized by oxygen very rapidly at high temperatures, yet no luminescence appears in the absence of luciferase; luciferin can also be oxidized rapidly by $K_3Fe(CN)_6$ in the presence of luciferase but absence of oxygen, yet no luminescence appears. Only if both oxygen and luciferase are present, is luminescence dependent on reaction velocity (10).

Luciferase apparently plays two rôles: (1) to catalyze the oxidation of luciferin and (2) to supply easily excited molecules (A′) which emit light (luminescence) on return to the normal state (A). Luciferin is a substance whose energy of oxidation excites the luciferase. We may imagine the steps in the process as follows:

$$\text{Luciferin } (LH_2) \rightleftarrows \text{oxyluciferin } (L) + H_2 - 15 \text{ calories}$$
$$H_2 + \tfrac{1}{2}\,O_2 = H_2O' + 69 \text{ calories}$$

where H_2O' represents an energy-rich water molecule.

$$H_2O' + \text{luciferase } (A) = H_2O + \text{excited luciferase } (A')$$
$$A' = A + hv$$

This view again leads to the question why other easily oxidized substances (like methylene white) cannot take the place of luciferin when oxidized in the presence of luciferase, provided their oxidation supplies sufficient energy. As a matter of fact it is impossible to obtain light on mixing luciferase with autooxidizable substances, although a large number has been tested. In reply we can only say that the excitation of luciferase is specific as so many other biological phenomena are specific where proteins (luciferase behaves like a protein) are concerned.

It is possible that specificity depends on adsorption. Oxyluciferin cannot be *completely* separated from luciferase after dialysis through parchment for 12 days, although much of the oxyluciferin will dialyze away. Luciferase cannot pass through parchment. Kautsky and Zocher found that fluorescent dyes adsorbed on siloxene surfaces were the ones that could be excited to luminesce. It is possible that the autooxidizable substances tested were not adsorbed by luciferase and so were unable to transfer their energy to the luciferase.

SUMMARY.

Oxidation of luciferin at anodes of various metals by nascent oxygen or oxidation by colloidal platinum or palladium and oxygen or other oxidizing agents never results in luminescence in the absence of luciferase.

Luciferase solution electrically connected with luciferin solution (by platinum electrodes in metallic contact and a salt bridge) shows no luminescence.

Luciferin or luciferase mixed with oxidized siloxene hydroxide and oxidized with acid permanganate does not luminesce. Fluorescent dyes do not luminesce in the presence of oxidizing luciferin alone or of luciferase alone.

Luciferin in alcohol luminesces slightly on heating or on addition of $KMnO_4$, disuccinyl peroxide, ozonized turpentine, and some other oxidizing agents. These are the only conditions in which I have ever observed luciferin luminescence without luciferase in solution.

Luciferase rather than luciferin is the source of the light in bioluminescence. The most probable hypothesis is that luminescence appears as a result of the excitation of luciferase molecules by the energy of oxidation of the luciferin. Luciferase plays in addition the part of catalyst, increasing the velocity of oxidation of luciferin. This hypothesis cannot be considered as proved until some oxidizing substance is found other than luciferin which can transfer its energy to luciferase with the production of light.

BIBLIOGRAPHY.

1. Dubois, R., *Ann. Soc. Linn. Lyons*, 1913, lx, 81; 1914, lxi, 247.
2. Harvey, E. N., *J. Gen. Physiol.*, 1918, i, 133.
3. Harvey, E. N. *J. Gen. Physiol.*, 1927, x, 385.
4. Harvey, E. N. *J. Gen. Physiol.*, 1923, v, 275.
5. Harvey, E. N. *J. Gen. Physiol.*, 1922, iv, 285; *Am. J. Physiol.*, 1926, lxxvii, 548.
6. Harvey, E. N. *Science*, 1917, xlvi, 241.
7. Harvey, E. N. *Am. J. Physiol.*, 1924, lxx, 619.
8. Kautsky, H., and Zocher, H., *Z. Physik*, 1922, ix, 267; *Z. Elektrochem.*, 1923, xxix, 308; *Naturwissenschaften*, 1923, xi, 194.
9. Harvey, E. N., *Am. J. Physiol.*, 1926, lxxvii, 555.
10. Amberson, W. R., *J. Gen. Physiol.*, 1922, iv, 517.

A COLORIMETRIC METHOD FOR THE DETERMINATION OF FREE TRYPTOPHANE IN BLOOD.

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For the study of certain problems in protein metabolism it is not sufficient to be able to determine the total free amino N in blood and body tissues; but it is highly desirable to follow, at least relatively, the concentration of some of the individual amino acids in this connection. No very satisfactory method of doing this has been available. Owing to the exceedingly small concentration of these substances in blood, colorimetric methods alone are applicable. Some methods of this sort have been used with protein hydrolyzates, but their application to so called protein-free blood filtrates is in general rendered either impossible or difficult by the presence of numerous interfering substances. Two attempts to apply available colorimetric methods in blood determinations may be noted.

Cary and Meigs (1) estimated the concentration of free tryptophane in blood by taking advantage of its well known reaction with *p*-dimethylamidobenzaldehyde. Owing to color differences between blood samples and standards, the method was not very satisfactory. The reaction also is given by indole derivatives in general. For the purpose of their work, it seemed justifiable to assume that the concentration of indole derivatives other than tryptophane in the mercuric sulfate precipitates from their concentrated deproteinized blood extracts could be disregarded, since the changes noted in the blood were large in comparison to the amount of non-tryptophane indole derivatives which it seemed reasonable to assume to be present. An investigation of this question, however, is desirable.

Another attempt to estimate the concentration of an amino

acid in blood was made by Harding and Cary (2). These investigators tried to apply to blood the Folin and Looney (3) method for the determination of cystine. They found very much more material giving this reaction in cow blood than it was anticipated could be present as cystine, and when cystine was added to the blood, it was not recovered quantitatively by any of the ordinary methods of deproteinization. It was finally concluded that the material in the blood giving the Folin and Looney reaction was glutathione. Holden (4) in the meantime had published evidence of the existence of glutathione in blood; and Hunter and Eagles (5) have since presented evidence of a quite different nature showing the absence of cystine in deproteinized blood extracts and the presence of either glutathione or some peptide very closely related to it, the nature of which is now under investigation. The attempt to determine cystine in blood by the Folin and Looney method, therefore met unexpected difficulties and even produced evidence which suggests that possibly little or no free cystine occurs in blood.

The present paper presents a very much more satisfactory procedure for the determination of protein-free tryptophane in blood than that used in the previous work in this laboratory. The procedure depends upon the familiar Hopkins-Cole reaction with glyoxylic acid and the oxidation of the tryptophane-glyoxylic acid condensation product by means of mercuric sulfate. The method, like that with *p*-dimethylamidobenzaldehyde, is not specific for tryptophane, but gives colors of varying character with other indole derivatives. Some evidence is presented in this paper to show that the indole material in the blood that is estimated by the method here used is largely tryptophane that is either free in blood or freed by the procedure from simple peptide combinations that may exist therein. It is felt that this method is suitable for practical routine determinations where blood samples of sufficient size are available.

Description of Method.

The procedure as finally adopted for the determination of the protein-free tryptophane in blood and plasma is as follows: Freshly drawn samples of blood[1] or blood plasma are coagulated

[1] To prevent coagulation 0.7 cc. of a sodium citrate solution (38 gm. of sodium citrate per 100 cc. of solution) per 100 cc. of blood were used.

with acetic acid, treated with kaolin, filtered, concentrated *in vacuo* (about 20 to 25 mm.) and treated again with kaolin. Protein-free aliquots are then taken out as in the amino N procedure in this laboratory (1, 6) except that no trichloroacetic acid is used.

These protein-free aliquots are diluted to a known volume of about 1½ to 2 times the blood or plasma represented by them. In doing this enough 33⅓ per cent H_2SO_4[2] is used to give 7 per cent by volume of concentrated acid in the final solution. The tryptophane is precipitated from this solution by dissolving in it 10 gm. of $HgSO_4$ per 100 cc. and allowing it to stand in an ice box from 4 to 48 hours. The precipitate is then filtered upon a fine asbestos mat. The original vessel and mat are washed three times, 10 cc. of 5 per cent (by volume) H_2SO_4 being used each time. The mat is again washed three times, and along with the precipitate, is returned to the original vessel. 20 cc. of freshly prepared glyoxylic-sulfuric acid reagent[3] are then poured through the Gooch filter into this vessel and 4 drops of a 25 per cent solution of $HgSO_4$ in 10 per cent (by volume) H_2SO_4 are added. The vessel is then stoppered and allowed to stand at room temperature, but not in direct sunlight, for about 48 hours, or in a well cooled ice box at about 5° for about 5 days, after which the development of the color of the solution is complete. The colored solutions are then filtered through dry asbestos mats and compared colorimetrically with tryptophane standards of suitable size prepared from a stock solution by similar precipitation and treatment.

The extent to which the color of blood samples and standards match is shown in Fig. 1, which gives the extinction coefficients at various wave-lengths of light as determined by means of a spectrophotometer for such a sample and standard, the color being developed in them at room temperature. Samples and standards of suitable size are readily matched in the colorimeter. They are

[2] 1 volume of concentrated H_2SO_4, diluted to 3 volumes with water, was used.

[3] The glyoxylic-sulfuric acid reagent contains 20 cc. of H_2SO_4 solution (stock solution containing 21 cc. of 95 per cent H_2SO_4 diluted with 5 cc. of water) and 1 cc. of glyoxylic acid solution (stock solution made by adding 6 gm. of 5 per cent sodium amalgam to 100 cc. of a saturated solution of oxalic acid and filtering after completion of the reaction).

blue or bluish violet. According to Fig. 1, the solution prepared from blood samples contains slightly more yellow than the standard, but this difference, when viewed by the naked eye or in the colorimeter, is generally slight. Small standards and samples likewise are perceptibly more yellow than large ones when observed colorimetrically. Perceptible, but negligible, differences of this sort may also sometimes be noticed between standards of the same size. Samples which have been kept in the ice box should be brought to room temperature before being compared colorimetrically.

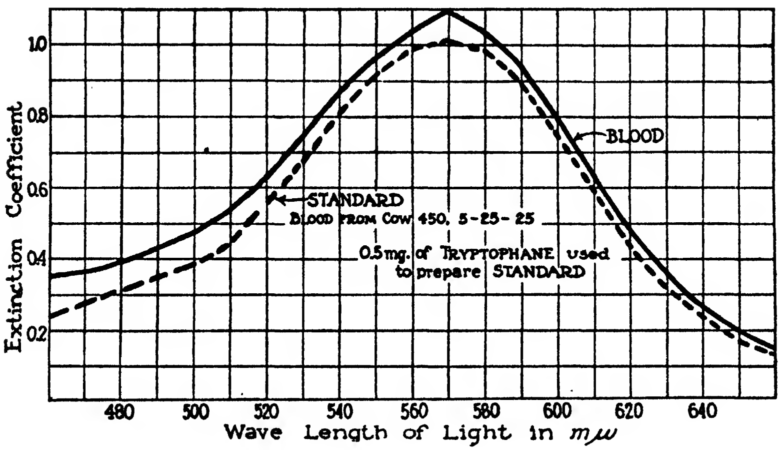

FIG. 1. Extinction coefficients at various wave-lengths of light for colored tryptophane-glyoxylic acid solutions prepared from pure tryptophane ("standard") and from an acid mercuric sulfate precipitate from a concentrated protein-free blood extract, the procedure of the tryptophane method described in text being used.

Application of Method to Known Quantities of Tryptophane.

Colorimetric results obtained by the above method from known quantities of tryptophane are shown in Table I. There is not a good agreement between some of the triplicate 0.2 mg. samples; but larger duplicates or triplicates agree fairly well. The agreement between the samples of June 16 and June 19, 1925 indicates that it is immaterial whether the color is allowed to develop at room temperature or at 5°, as described above. This is confirmed by

other work which will be cited later. Hence, all results in this table from samples of the same size may be averaged. When this is done it appears from the column "Uncorrected results" that the smaller samples read as if they contain relatively less tryptophane than was initially used in their preparation. The 0.5, 0.3, and 0.2 mg. samples are 2.7, 6.7, and 11.9 per cent lower respectively than would thus be calculated for them.

These data suggest that a constant loss of approximately 0.035 mg. of tryptophane per sample occurred in these determinations. It will be shown later that with blood samples of constant size there is a loss in chromogenic value proportional to the volumes used in the acid mercuric sulfate precipitation; whereas, with samples of varying size, precipitated in volumes that are proportionate, the determinations agree well. These facts are taken to mean that tryptophane has a definite and perceptible solubility in these acid mercuric sulfate solutions, which for pure tryptophane precipitated as above is approximately 0.035 mg. per 100 cc. When the uncorrected results in Table I are increased accordingly, as shown in the column "Result corrected for precipitation loss," the colorimetric value of tryptophane samples is closely proportional to the calculated quantity of this amino acid present. This correction, therefore, should be made with all standards used in the above described method for the determination of tryptophane in blood. The standards may then be precipitated in any convenient volume, be directly compared colorimetrically even when as much as 75 per cent apart in their tryptophane content (a difference of this magnitude should be avoided if possible), and yield results in good agreement with the actual tryptophane present provided this is 0.265 mg. or more.

The results with the samples of June 16 and June 19, 1925 also tend to indicate that no changes occur in the chromogenic value of tryptophane solutions when they are kept for several days in an ice box even though no preservative is added. Other results accord with these. A tryptophane solution which had been kept thus for 8 months showed a decrease of only 4 per cent, whereas one which had been kept 3 months dropped 10 per cent in chromogenic value when compared with a freshly prepared solution. For many practical purposes, therefore, stock tryptophane solutions may be kept for some time in an ice box at approxi-

TABLE I.

Colorimetric Relations Found between Samples Which Initially Contained Known Quantities of Tryptophane.

The averages of the 0.7 mg. samples are taken as 100.

Date of sample.	Size of sample initially taken. 0.2 mg. Uncorrected results.	0.2 mg. Results corrected for precipitation loss.	0.3 mg. Uncorrected results.	0.3 mg. Results corrected for precipitation loss.	0.5 mg. Uncorrected results.	0.5 mg. Results corrected for precipitation loss.	0.7 mg. Uncorrected results.	0.7 mg. Results corrected for precipitation loss.
1925								
June 16*	26.9	31.0	40.3	43.3	70.7	72.2	99.4	99.4
" 16	26.8	30.8	41.4	44.5	73.5	75.0	99.4	99.4
" 16	26.5	30.5	40.0	43.0	70.0	71.5	98.7	98.7
" 24*	25.6	29.5	40.3	43.3	69.8	71.3	100.4	100.4
" 24	24.5	28.2	39.1	42.0	70.6	72.1	100.4	100.4
" 24	25.2	29.0	39.2	42.1	68.2	69.6	98.6	98.6
" 19†	27.2	31.3	41.1	44.2	69.3	70.8	101.4	101.4
" 19	25.8	29.7	41.4	44.5	69.3	70.8	101.1	101.1
" 19	24.5	28.2			69.0	70.5		
" 29†	24.1	27.7	39.0	41.9	69.5	71.0	100.4	100.4
" 29	22.1	25.5	38.6	41.5	67.2	68.6	100.4	100.4
	23.0	26.5	39.0	41.9	65.9	67.3	99.7	99.7
Mean for ice box samples...........	25.9		40.1		70.5		99.5	
Mean for room temperature samples..	24.4		39.8		68.4		100.6	
Mean for all samples.	25.2	29.0	40.0	42.9	69.5	70.9	100.0	100.0
Relation between initial quantities used..............	28.6		42.9		71.4		100	

The samples were precipitated by acid mercuric sulfate, a volume of 100 cc. being used. The color was developed as in the method described in the text. Those samples most nearly alike in the quantity of tryptophane were directly compared in the colorimeter. The samples of June 16 and June 19, 1925 were directly compared, and therefore are all expressed in

mately 5°. A solution containing 0.1 mg. per cc. is very convenient.

That these stock solutions frequently undergo chemical changes which are actually much greater than these colorimetric results indicate, is shown by the spectrophotometric determinations which will now be considered. Determinations of this sort were made with a number of the samples in Table I. The extinction coefficients for these samples and others, in which known quantities of tryptophane were used are shown in Table II. The readings were made at 560, 570, and 580 $m\mu$ and the figure given is the average of these results. These data are not presented to establish a spectrophotometric method that may be used to determine tryptophane in routine analyses, but rather because they bear upon the interpretation of the colorimetric results obtained, and especially upon the nature of the chromogenic material in blood that is estimated as tryptophane by the colorimetric method described above.

These spectrophotometric data accord with the colorimetric results in showing that the agreement between duplicate or triplicate determinations is in general very satisfactory when the tryptophane finally present is 0.265 mg., or more, and that it is immaterial whether the color is allowed to develop at room temperature or in an ice box at 5°. Likewise the 0.5 and 0.7 mg. samples of June 16 and June 19, 1925 show the same agreement as was noted colorimetrically. On the other hand, a comparison of the samples from the stock tryptophane solution that was made

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relation to the average of all the 0.7 mg. samples of these dates. As no difference was noted between the samples in which the color developed in the ice box (*) and those in which it developed at room temperature (†) the same procedure was followed in expressing the results of June 24 and June 29, 1925 although none of the former was compared with the latter. In the columns headed "Results corrected for precipitation loss," a loss equivalent to 0.035 mg. of tryptophane per sample is assumed. The samples of June 16 and June 19, 1925 were taken from a tryptophane solution made up June 16, and those of June 24 and June 29, 1925 from a solution made up June 24. These solutions were kept in an ice box without addition of preservative. All of the determinations, except some of those of June 19, 1925, were run in triplicate.

* Color developed while sample was in the ice box.

† Color developed at room temperature.

TABLE II.

Extinction Coefficients for Samples Prepared from Known Quantities of Tryptophane.

Date of sample.	Tryptophane initially used to prepare sample.							
	0.2 mg.		0.3 mg.		0.5 mg.		0.7 mg.	
	Tryptophane finally present, allowing for loss in precipitation.							
	0.165 mg.		0.265 mg.		0.465 mg.		0.665 mg.	
	Average of extinction coefficients determined at 560, 570, and 580 $m\mu$.							
	Samples from unchanged stock solution.	Samples from changed stock solution.	Samples from unchanged stock solution.	Samples from changed stock solution.	Samples from unchanged stock solution.	Samples from changed stock solution.	Samples from unchanged stock solution.	Samples from changed stock solution.
1925								
June 24 (ice box)*..........	0.343		0.597		1.098		1.71	
" 24 " " *..........	0.288		0.550		1.121		1.74	
" 24 " " *..........	0.331		0.567		1.137		1.66	
" 29 (room temperature)*		0.311		0.538		0.996		1.54
" 29 " " *		0.287		0.514		0.980		1.55
" 29 " " *		0.270		0.522		0.945		1.47
May 25 " " †						0.992		
June 16 (ice box)‡..........	0.405		0.608		1.15		1.76	
" 19 (room temperature)‡	0.444		0.679		1.12		1.76	
July 8 " " *						0.864		
" 8 " " *						0.910		
" 8 (ice box) *..........						0.931		
" 8 " " *..........						0.899		
" 8 " " *..........						0.881		
Average..................	0.362	0.289	0.600	0.525	1.125	0.933	1.73	1.52
$\frac{\text{Average}}{T}$ §..................	0.181	0.145	0.200	0.175	0.225	0.187	0.247	0.217
$\frac{\text{Average}}{T'}$ ¶..................	0.219	0.175	0.226	0.198	0.242	0.201	0.260	0.229

All stock tryptophane solutions here used were kept in an ice box at approximately 5° and without preservative. The tryptophane was precipitated in a 100 cc. volume, the color developed with glyoxylic acid, *etc.*, as described in the text, and the coefficients determined at 560, 570, and 580 $m\mu$. The average of these is given.

* From stock tryptophane solution made June 24, 1925.

† From stock tryptophane solution made February 2, 1925.

‡ From stock tryptophane solution made June 16, 1925.

§ Tryptophane originally in sample.

¶ Tryptophane finally present after allowing for loss in mercuric sulfate precipitation.

up June 24, 1925 shows clearly that as time went on there was a decided change in its chromogenic value as determined spectrophotometrically; but, whereas the samples of June 29, 1925 are 10 per cent lower and those of July 8, 1925 20 per cent lower than those of June 24, 1925 from the same stock tryptophane solution, the chromogenic value of this solution when viewed colorimetrically after the end of 3 months had dropped only 10 per cent. Likewise on May 5, 1925 a sample from the stock tryptophane solution made up February 2, 1925, gave an extinction coefficient apparently 12 per cent low, whereas on October 2,

TABLE III.

Calculated Extinction Coefficients of Tryptophane Samples as Compared with Observed Mean.

Tryptophane in sample, *mg*	0.1	0.165	0.2	0.265	0.3	0.4	0.465
Calculated extinction coefficient	0.214	0.362	0.444	0.602	0.690	0.952	1.131
Observed extinction coefficient (mean)		0.362		0.600			1.125

Tryptophane in sample, *mg*	0.5	0.6	0.665	0.7	0.8	0.9	1.0
Calculated extinction coefficient	1.230	1.524	1.724	1.834	2.160	2.502	2.860
Observed extinction coefficient (mean)			1.73				

Data are based on quantity of tryptophane in samples after allowing for the loss in mercury precipitation. Only samples from unchanged tryptophane solutions were used.

1925 samples that were read colorimetrically ran only 4 per cent low. It has not been determined whether or not this change occurs in tryptophane solutions containing dilute sulfuric acid as recommended by Folin and Looney (3). The extinction coefficients in the column "Samples from unchanged stock solution" in Table II are apparently those for freshly prepared solutions.

After the development of color in some of these tryptophane-glyoxylic acid solutions was complete, they were diluted with sulfuric acid of the same strength. The relation between the absorption of light and the dilution of the solution when measured either colorimetrically or spectrophotometrically accorded with Beer's law. Nevertheless, it will be noted in Table II that the

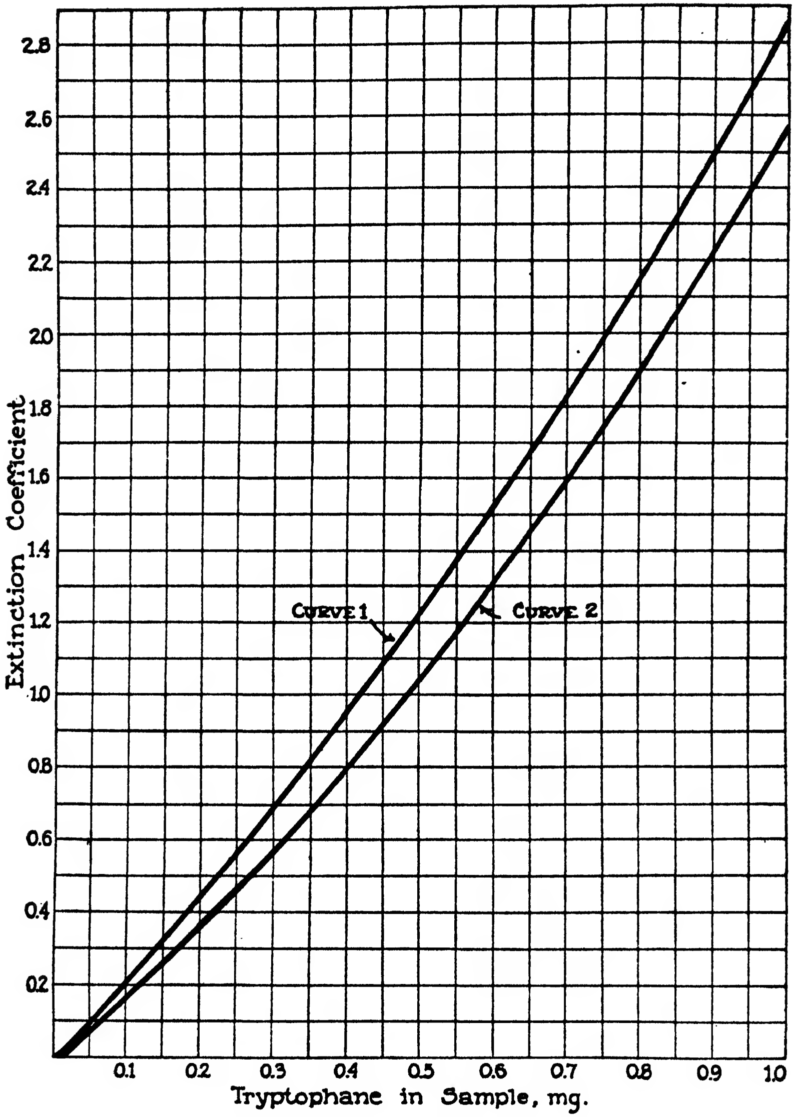

FIG. 2. Average of extinction coefficients at 560, 570, and 580 $m\mu$ for samples containing known quantities of tryptophane. Color developed as in method in text. Curve 1 for samples taken from freshly prepared tryptophane solutions; Curve 2 for samples from tryptophane solutions which appear to have altered since their preparation. The curves express the tryptophane actually present in the sample after allowing for loss in mercury precipitation.

extinction coefficients are not exactly proportional to the tryptophane actually present in these samples. For each 0.1 mg. of tryptophane in a sample from tryptophane solutions which had undergone no change in their chromogenic value since their preparation, the extinction coefficient apparently increases on an average approximately 0.008 with each increment of 0.1 mg. in

TABLE IV.

Colorimetric Results with Aliquots from Same Concentrated Deproteinized Blood Extract.

Cow 450, April 15, 1925.

Sample No.	Blood represented in sample.	Precipitation volume.	Precipitation volume / Blood volume.	Tryptophane in blood. Uncorrected results.	Tryptophane in blood. Results corrected for precipitation loss.
	cc.	*cc.*		*mg. per 100 cc.*	*mg. per 100 cc.*
1, 2	44	30	0.68	1.03	1.05
3, 4	44	50	1.13	0.96	1.00
5, 6	44	75	1.70	0.95	1.01
7, 8	44	100	2.27	0.92	1.00
9, 10	44	150	3.41	0.92	1.03
11, 12	44	200	4.55	0.88	1.03
13, 14	35.2	80	2.27	0.93	1.01
15, 16	26.4	60	2.27	0.95	1.03
17, 18	17.6	40	2.27	0.94	1.02

The color in all of these samples developed at room temperature. A 0.5 mg. standard was used. A blood sample of nearly the same colorimetric value was matched with it, and the blood samples were then compared with one another. Each result in the fifth column represents the average of two determinations. The standard has been corrected for loss of tryptophane in mercury precipitation. In the column giving uncorrected results no allowance of this sort is made for loss in precipitation of blood samples; whereas in the column giving results corrected for precipitation loss, this loss is assumed to be the same as with standards.

the size of the sample. This increase was fairly constant. One may, therefore, calculate the mean extinction coefficient for a 0.1 mg. sample and for samples of various sizes. This has been done in Table III, and it will be noted that the mean observed and calculated coefficients agree very well. Curve 1, (Fig. 2) is drawn in accord with these calculated data, only samples from unchanged tryptophane solutions being used. Curve 2 is similarly

TABLE V.

Agreement of Duplicate Colorimetric Determinations of Tryptophane in Cow Blood and Plasma.

Sample No.*	Date.	Tryptophane in blood and plasma.	
		Individual determinations.	Average.
	1925	*mg. per 100 cc.*	*mg. per 100 cc.*
B-J 450	Apr. 15	1.01, 1.01, 0.99, 1.00, 1.03, 1.04, 1.04, 1.03, 1.00, 1.02, 1.04, 1.03, 1.01, 1.02	1.02
B-J 450	May 25	1.32, 1.38	1.35
B-J 49	July 3	1.22, 1.23, 1.23	1.23
P-J 7	" 18	0.97, 0.99, 1.00, 0.97	0.98
P-M 7	" 18	0.84, 0.79, 0.81, 0.81	0.81
P-J 414	Aug. 7	1.31, 1.29, 1.27	1.29
P-M 414	" 7	1.26, 1.31, 1.26	1.28
P-J 7	" 28	1.24, 1.27	1.26
P-M 7	" 28	1.02, 1.06	1.04
P-J 414	Sept. 10	1.29, 1.33	1.31
P-M 414	" 10	1.19, 1.21	1.20
P-J 33	" 21	1.23, 1.23	1.23
P-M 33	" 21	1.19, 1.21	1.20
P-J 33	July 29	0.89, 0.97, 0.93, 0.95	0.94
P-M 33	" 29	0.88, 0.83	0.86
Mixed plasma.†	Oct. 14	1.23, 1.19, 1.24	1.22

The color developed at room temperature. All samples are corrected for loss of tryptophane in mercury precipitation. With the first three blood samples, aliquots from the same protein-free extracts were used in each case. The table includes all samples made by the procedure described in the text, which was finally adopted for routine work April 15, 1925.

* B = determinations made on whole blood.
P = " " " blood plasma.
J = samples obtained from jugular vein.
M = " " " abdominal subcutaneous (mammary) vein.
Figures refer to the number of the cow from which the sample was obtained.

† Plasma from several cows.

derived from the results with samples from solutions which apparently had changed in chromogenic value since their preparation. In both cases the extinction coefficient is expressed for the tryptophane actually present in the sample after allowing for the loss in mercury precipitation.

Application of Method to Blood.

In Table IV are shown the colorimetric results obtained when the method described above for the determination of tryptophane in blood is applied to different aliquots from the same concentrated protein-free blood extracts. The aliquots represent from 17.6 to 44 cc. of blood. From the column "Uncorrected results" in which no allowance is made for the loss of blood tryptophane in mercury precipitation, two conclusions may be drawn. First, when the size of the samples is constant, the tryptophane when calculated thus per 100 cc. of blood is lower in proportion to the volume used in the acid mercuric sulfate precipitation; and secondly, when the blood samples vary in size and these precipitation volumes vary proportionately, the results agree well.

It is obvious therefore that some correction must also be made for the loss of blood tryptophane in this precipitation. Onslow (7) showed that the precipitability of various amino acids by acid mercuric sulfate is influenced by the presence of other amino acids. The data in the column "Results corrected for precipitation loss" indicate, first, that under the conditions which have been given above for this precipitation, the same correction applies as with the standards; secondly, that all standard and blood results should be corrected accordingly; and, finally, that blood samples may vary in size within the limits here compared and show excellent agreement. The samples in which the mercury precipitation volumes were 0.68 and 1.13 times the blood represented were somewhat yellower than the other samples of the same size. Hence, larger precipitation volumes than these are preferable. The washing of the blood precipitates with 5 per cent H_2SO_4 also tends to improve the color; but, if carried to excess, leads to a perceptible loss of tryptophane. In the method, as described, this loss is apparently negligible.

Table V shows the agreement found between repeated colorimetric determinations of tryptophane in blood and blood plasma

according to the method described above. The average of the mean deviations with the various blood samples is 0.015 mg., or 1.4 per cent; and the extreme deviation of any determination from the mean for the corresponding blood sample is 0.04 mg., or 4.3 per cent. This agreement in duplicate determinations is certainly very satisfactory.

In the case of the blood, Sample B-J 49, of July 3, 1925, shown in Table V, six additional determinations were made in which the color was developed at approximately 5° (ice box). They were compared with standards that were similarly handled, and gave

TABLE VI.

Recovery of Tryptophane Added to Blood or Blood Plasma Just Prior to Coagulation in the Method for Determining Tryptophane Described in the Text.

Sample.	Tryptophane found in sample.		Recovery.	Tryptophane added.	Recovery.
	Originally.	With tryptophane added.			
	mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*
Blood, Cow 42, Jan. 10, 1925.*	1.05	1.96	0.91	0.94	96.8
Blood plasma, mixed sample from several cows, Oct. 14, 1925.†	1.26	2.08	0.82	0.83	98.8

* Method used was practically the same as that finally adopted.

† In some of the determinations uranium acetate was used as a protein precipitant after coagulation with acetic acid.

an average of 1.23 mg. of tryptophane per 100 cc. of blood—the same as the average of the three determinations in Table V, where the color was developed at room temperature. The two procedures, therefore, give practically the same results with the blood just as they do with standards.

Two experiments were carried out to determine whether tryptophane added to cow blood or blood plasma immediately before coagulation is recovered quantitatively by the method described above. These data are given in Table VI. The recovery is certainly all that could be expected, and is *prima facie* evidence that the chromogenic value of tryptophane in blood, when thus

measured, is not materially affected in the process by the other constituents present or by the procedure used in preparing the protein-free extracts. This, however, cannot be taken as final evidence that tryptophane passes entirely unaltered through these processes.

Experiments have been tried to determine whether the tryptophane combined as protein and polypeptides in blood could be more completely precipitated by other methods than by that described in this article. Not all of these experiments have yielded definite results because the final solutions were frequently too different in color to be compared. This was true with colloidal ferric hydroxide and tungstic acid. Trichloroacetic acid, if not removed from solution, also may give a voluminous precipitate of mercurous chloride (8) in the acid mercuric sulfate precipitation. This interferes with the final color; but this condition may be avoided, as frequently the tryptophane precipitation is complete before any of the mercurous chloride comes down. The effect has therefore been studied of adding 10 per cent of trichloroacetic acid to the concentrated blood extracts just prior to the second treatment with kaolin, proceeding as in the method used for the determination of amino N in this laboratory (6, 1). The trichloroacetic acid was not destroyed; and, with the results here cited, there was no evidence of mercurous chloride precipitation when the tryptophane-mercuric sulfate precipitate was filtered off. Three determinations made thus on the same blood gave 1.00, 0.97, and 0.95 mg. per 100 cc. respectively, or an average of 0.97 mg.; whereas the usual method of deproteinization gave 1.00 and 0.95 mg. or practically the same average. The color of all of these was good. Some plasma determinations were also carried out in which 50 cc. aliquots (equivalent to 60 cc. of plasma) were taken from the same protein-free extract, and treated with varying amounts of uranium acetate (1, 5, and 10 cc., of a 10 per cent solution). After the precipitates were filtered off, the filtrates were treated with an excess of N NaOH, filtered, and these filtrates precipitated as usual with sulfuric acid and mercuric sulfate. The results thus obtained were 1.28 and 1.32 mg. per 100 cc. of plasma. With aliquots that were not thus treated with uranium acetate the results were 1.26, 1.24, and 1.21 mg. per 100 cc., or even less, on an average, than with the uranium acetate

determinations. It is, therefore, evident that the method of deproteinization recommended above is satisfactory for the determination of free tryptophane in blood and plasma.

To What Extent Is Material Estimated in Blood by the Above Method Actually Tryptophane?

The fact that the correction for the solubility in acid mercuric sulfate is the same with both tryptophane standards and blood

TABLE VII.

Tryptophane in Blood of Cow 49, July 3, 1925, According to Samples Read Both Colorimetrically and Spectrophotometrically, 560, 570, and 580 mμ Being Used.

Sample No.	Temperature at which color developed.	Tryptophane per 100 cc. of blood.		
		Spectrophotometric results.		Colorimetric results.
		mg.*	mg.†	mg.
I-70-a	Room temperature.	0.95	1.07	1.23
I-90-a	" "	0.97	1.10	1.22
I-40	" "	0.91	1.07	1.26
I-60	Ice box.	0.94	1.06	1.32
I-80	" "	0.96	1.06	1.22
II-40	" "	0.90	1.04	1.19
II-60	Room temperature.	0.93	1.04	1.20
II-80	" "	0.91	1.01	1.19
Average		0.93‡	1.06§	1.23

* Calculated according to Fig. 2, Curve 1.
† Calculated according to Fig. 2, Curve 2.
‡ This is 24 per cent lower than the average colorimetric result.
§ This is 14 per cent lower than the average colorimetric result.

samples is interesting as tending to indicate the identity of the chromogenic material involved. It will be shown in another article (9) that this material in blood is used to some extent by the mammary gland in milk secretion, and therefore unquestionably contains tryptophane. To determine to what extent this material is tryptophane, a number of experiments has been carried out. The first that will be considered in this connection are those in which it was attempted to determine the tryptophane in blood spectrophotometrically, and to compare the light absorption of blood samples and standards.

Eight of the blood samples of Cow 49, July 3, 1925, were read in the spectrophotometer as well as in the colorimeter. The extinction coefficients were determined at 560, 570, and 580 $m\mu$, and the tryptophane calculated from the curves in Fig. 2. These results are shown in Table VII. They agree well among themselves when similarly calculated; but on an average are either 14 per cent (Curve 2, Fig. 2 being used) or 24 per cent (Curve 1, Fig. 2 being used) lower than the colorimetric figure. When the tryptophane in the sample of blood for which the absorption curve is shown in Fig. 1 is calculated in accord with the curves in Fig. 2, similar differences are found between the spectrophotometric

TABLE VIII.

Spectrophotometric Determination of Tryptophane in Blood of Cow 450, May 25, 1925, Determined at Various Wave-Lengths.

Wave-lengths used in determination and method of calculation.	Mg. per 100 cc. of blood.	Difference from same sample read colorimetrically.
		per cent
Using 560, 570, and 580 $m\mu$ and Fig. 2, Curve 1........	1.06	23
" 560, 570, " 580 " " " 2, " 2........	1.20	13
" 560, 570, " 580 " " standard in Fig. 1....	1.18	14
" 460–660 $m\mu$ and standard in Fig. 1............	1.22	12
" 530–660 " " " " " 1...........	1.19	14
" 460–520 " " " " " 1...........	1.35	2.2
According to colorimetric reading..................	1.38	

and colorimetric results. This is shown in Table VIII. On the other hand, if the tryptophane in this sample of blood is calculated from the absorption curve of the standard, which is also shown in Fig. 1, the spectrophotometric results may or may not agree with those determined colorimetrically, depending upon the wave-lengths used in the calculation.

When these absorption curves in Fig. 1 are inspected, certain additional facts are evident. First, throughout the portion of the spectrum between 530 and 660 $m\mu$ the ratios between the corresponding absorption coefficients for the blood sample and standard are practically constant; secondly, between 460 and 520 $m\mu$ this is not true; and, finally, the maximum absorption in each case

is at exactly the same wave-length—a wave-length which comes within the first mentioned range. These facts cannot be taken as final proof but certainly tend to indicate, first, that in the main, one and the same colored substance in this blood and standard determined the absorption in the spectrum betweeen 530 and 660 $m\mu$; secondly, that at least one other colored or chromogenic material was present in the blood, led to a yellow pigment in the final colored solution, and thereby affected the spectrophotometric readings between 460 and 520 $m\mu$ and the colorimetric results, but did not appreciably affect the extinction coefficients between 530 and 660 $m\mu$; and, finally therefore that about 86 per cent of the color measured colorimetrically in this sample of blood is due to the same chromogenic material as occured in this standard. This reasoning would lead to a similar conclusion with the blood samples from Cow 49. Attention, however, should here be drawn to the fact that the standard in Fig. 1 had an average extinction coefficient at 560, 570, and 580 $m\mu$ of 0.99 instead of 1.12 for similar standards prepared from fresh tryptophane solutions.

That the tryptophane finally estimated in blood may be 15 to 25 per cent less than that determined colorimetrically and that the rest of the color thus estimated is due to some yellow pigment is confirmed in another way. A yellowish material was constantly noted in the concentrated protein-free blood extracts and on the asbestos mats after filtering the mercury precipitates. This could be precipitated from these blood extracts by means of either neutral or basic lead acetate, or could be removed by extraction with amyl alcohol, toluene, or ether. When thus removed from a concentrated blood plasma extract by means of lead acetate, the tryptophane, as determined colorimetrically, was reduced from 1.24 mg. per 100 cc. of plasma to 1.06 mg., or 14 per cent. Samples of blood when similarly treated dropped in one case from 1.01 to 0.87 mg., or 14 per cent, and in another from 1.14 to 0.85 mg., or 25 per cent. Extraction of these concentrated protein-free preparations with the solvents which were found to remove this yellow material also reduced the tryptophane as determined colorimetrically. Aliquots from the above plasma extract were thus extracted four times with toluene. In one instance this was done just before adding the sulfuric acid and in another immediately thereafter. The results were respectively 1.09 and 1.04 mg.

Other aliquots similarly treated with ether instead of toluene gave 0.94 and 0.95 mg. respectively. This plasma, therefore, which gave 1.24 mg. per 100 cc. when treated by the usual method, gave 1.06 mg., or 14 per cent less, when treated as above with lead acetate or with toluene, and 0.95 mg., or 23 per cent less, when treated with ether.

Similar methods of treatment were tried with the blood of Cow 450 for which the absorption curve was taken to indicate that 86 per cent of the color as read colorimetrically was due to the same chromogenic material as occurred in the standard used. The average of the colorimetric samples of this blood, when treated as usual, was 1.35 mg. per 100 cc., but a sample that was precipitated as above with lead acetate and subsequently extracted three times with amyl alcohol, twice with toluene, and four times with ether gave 1.09 mg., or 19 per cent less. A tryptophane solution when similarly treated lost 4.3 per cent, making a net drop of 15 per cent in the blood sample as a result of the removal of non-tryptophane material. This experiment, on the one hand, leaves no doubt that the actual tryptophane in the mercury precipitates with this blood was certainly 15 per cent less than the colorimetric results would indicate; and on the other hand, with the spectrophotometric results above, strengthens greatly the view that the other 85 per cent is actually tryptophane. Since this experiment also indicates that in the main the same non-protein material that affects the results is removed by these various processes, it may be concluded that the tryptophane in the mercury precipitates is in general about 15 to 25 per cent less than the colorimetric results by the usual method would indicate.

Whether this means that the actual tryptophane originally present in blood is thus 15 to 25 per cent less than the colorimetric results would indicate, depends upon the origin and nature of this non-tryptophane material. First, the results indicate that it occurs in the glyoxylic-sulfuric acid solutions as a yellow pigment; secondly, a yellow pigment occurs in the protein-free blood extracts and mercury precipitates; and finally, the methods that remove non-tryptophane material that interferes with these determinations, also remove the yellow pigment in these blood extracts. Although the resultant color in the glyoxylic-sulfuric

acid solutions has not been analyzed to show that the yellow pigment there is also removed, and that the absorption from 530 to 660 $m\mu$ is practically unaffected, it is believed that the evidence warrants the conclusion that the yellow pigment in the concentrated protein-free extracts is the main cause of the difference between the actual tryptophane in the mercury precipitates and that found ordinarily by the colorimetric method. It is still uncertain, however, whether it occurs unchanged in the glyoxylic-sulfuric acid solutions or whether it reacts with the glyoxylic acid to produce the pigment that affects the results. The latter view seems the more probable because its effect upon the results seems greater than one would otherwise anticipate.

The amount of yellow pigment in the protein-free extracts varied under different conditions. It was especially reduced by concentration *in vacuo* instead of at room temperature. It, therefore, does not appear to be solely an unremoved pigment that was present in the original blood; but, rather, to be formed to some extent in the process of concentration. The blood filtrates before concentration appear to be entirely colorless. Hopkins and Cole (10) noted the formation of such a pigment in the concentration of impure tryptophane solutions, and also precipitated it by means of normal lead acetate. Gortner and Blish (11) showed that under somewhat different conditions of heating and acidity tryptophane takes part in humin formation. The yellow material that is formed in the concentration of the blood extracts is not humin but may be a colored indole derivative formed from tryptophane originally present in the blood. It may be that a method eventually will be evolved to prevent its formation. In the meantime the spectrophotometric results, 530 to 660 $m\mu$ being used, or the results obtained by treating the protein-free extracts with lead acetate or various solvents, may be taken as minimum figures for the non-protein tryptophane in blood either free or possibly in simple peptide combinations that are split by the procedure used; whereas, the colorimetric results may be a fairly close approximation to the tryptophane in the original blood and be used in routine determinations of this sort. On the other hand, other indole derivatives than tryptophane may be the source of at least a portion of the difference between these two sets of results, but

would hardly affect those which would thus be taken as minimum figures.

The urine from some of the cows gave a very intense indoxyl reaction when extracted with chloroform after treatment with hydrochloric acid and copper sulfate. Haas (12) found that indoxyl derivatives are regular constituents of blood. 10 cc. of a protein-free blood filtrate, which was equivalent to 56 cc. of blood, were tested for material of this sort by extraction with chloroform after treatment with 1 cc. of a 10 per cent thymol solution and 10 cc. of Obermayer's reagent. The test was entirely negative. According to Jolles (13), this test, as carried out, is sensitive to 0.0032 mg. of urine indican. It is, therefore, certain that the quantity of this material in these protein-free extracts is negligible, although very plentiful in urine.

SUMMARY.

A method is described in this paper for the determination of non-protein tryptophane in blood and blood plasma by means of the Hopkins-Cole glyoxylic acid reaction in the presence of an excess of mercuric sulfate. The method is applied to mercury precipitates from deproteinized blood extracts. Correction must be made for the loss of the tryptophane in this precipitation. When this is done, good agreement is obtained with standards containing 0.265 mg. or more of tryptophane, and with blood or blood plasma samples of 25 cc. or more. Tryptophane added to the blood is recovered quantitatively. When determined colorimetrically the colors of the blood and standard match readily; but when analyzed spectrophotometrically this agreement is found not to be perfect. Evidence is presented to show that 75 to 85 per cent of the material in the blood that is finally estimated colorimetrically as tryptophane, according to the method described, is actually such, either being free or derived from simple peptides in the blood; but that 15 to 25 per cent of it is not. This material, however, may be derived in part from tryptophane originally present in the blood; so that the colorimetric method as described gives a fairly close approximation to the actual tryptophane present.

The spectrophotometric determinations in this paper were carried out by W. C. Holmes of the Color Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington. He also rendered valuable assistance in interpreting the results which he obtained. The writer desires to express his indebtedness to him and appreciation of his hearty cooperation. Thanks are also due to Dr. J. A. Ambler who was at that time in charge of the Color Laboratory.

BIBLIOGRAPHY.

1. Cary, C. A., and Meigs, E. B., *J. Agric. Research*, 1924, xxix, 603.
2. Harding, T. S., and Cary, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 319.
3. Folin, O., and Looney, J. M., *J. Biol. Chem.*, 1922, li, 421.
4. Holden, H. F., *Biochem. J.*, 1925, xix, 727.
5. Hunter, G., and Eagles, B. A., *J. Biol. Chem.*, 1927, lxxii, 133.
6. Cary, C. A., *J. Biol. Chem.*, 1920, xliii, 477.
7. Onslow, H., *Biochem. J.*, 1921, xv, 392.
8. Kharasch, M. S., and Chalkley, L. Jr., *J. Am. Chem. Soc.*, 1921, xliii, 607.
9. Cary, C. A., and Meigs, E. B., *J. Biol. Chem.*, 1928, lxxviii, 399.
10. Hopkins, F. G., and Cole, S. W., *J. Physiol.*, 1901–02, xxvii, 418.
11. Gortner, R. A., and Blish, M. J., *J. Am. Chem. Soc.*, 1915, xxxvii, 1630.
12. Haas, G., *Deutsch. Arch. klin. Med.*, 1916, cxix, 176.
13. Jolles, A., *Z. physiol. Chem.*, 1915, xciv, 79.

THE FREE TRYPTOPHANE IN COW BLOOD AND ITS UTILIZATION IN MILK SECRETION.

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In previous work (1) the present authors found that the free tryptophane of cow blood may vary independently of the free amino N. In one case when the free tryptophane of the blood dropped from 1.46 mg. to 0.63 mg. per 100 cc., or 57 per cent, the decrease in free amino N was only 14 per cent; and when the blood tryptophane increased from 0.63 mg. to 1.20 mg., or 90 per cent, there was no increase in blood amino N. In another case the free tryptophane of blood increased 100 per cent without significant change in the blood amino N. Changes of this sort in the concentration of free tryptophane in blood were brought about by changes in either the quantity or quality of protein in the diet.

This paper presents data on the concentration of free tryptophane in the blood and blood plasma of cows on diets which may be considered approximately standard, and also data on the utilization of this tryptophane by the mammary gland in the secretion of milk. The method of determining the concentration of tryptophane in the blood and plasma is described by Cary (2). The method of demonstrating its utilization in milk secretion is similar to that used first by Kaufmann and Magne (3), to show that the mammary gland uses glucose of the blood as the source of milk sugar, and later by Meigs, Blatherwick, and Cary (4), and by Cary (5) to show the utilization of the phosphatide and amino acids in the plasma of the blood in the synthesis of the fat and protein respectively of milk. The method is fully discussed in these references, and especially in a review by Meigs (6). As applied in the present paper, it consists in comparing the free tryptophane content of samples of blood taken simultaneously from the jugular and abdominal

subcutaneous (mammary) veins of milking cows, the blood from the former vein being assumed to represent approximately the tryptophane content of arterial blood and the difference between the two to represent the tryptophane removed from the blood by the mammary gland for use in milk secretion. Non-lactating animals served as controls. Since the glucose, phosphatide, and amino N used in milk secretion were found to be taken entirely from the plasma of the blood, the tryptophane in the present work

TABLE I.

Free Tryptophane in Cow Blood.

All of the samples were taken from the jugular vein.

Cow No.	Date of sample.	Tryptophane in blood.*
		mg. per 100 cc.
421	Dec. 20, 1924	0.95
	1925	
421	Jan. 10	0.99
421	" 28	1.02
421	Feb. 17	1.04
421	Apr. 1	1.16
458	Feb. 9	1.07
93	Mar. 13	1.47
450	" 23	0.98
450	Apr. 15	1.02
450	May 25	1.35
49	July 3	1.23

* Each figure is the average of several determinations. In some the method was not precisely that described, but gave practically the same results.

was determined only in the plasma. This was obtained by centrifuging immediately the fresh samples of blood at 3000 to 3600 R. P. M.

Before taking the samples of blood a halter and an antikicking device were placed on the cows. A cannula was then inserted into one of the jugular veins and held while another was inserted into a mammary vein. Collection of the blood was begun simultaneously from these two veins.

Table I gives the concentration of free tryptophane found in cow blood under the conditions already described. It varies from approximately 1 mg. to 1.5 mg. per 100 cc., and averages 1.12 mg.

TABLE II.

Free Tryptophane in Plasma from Cow Blood.

Samples from only the jugular vein are included.

Cow No.	Date of sample.	Tryptophane in plasma.
	1925	*mg. per 100 cc.*
421	Jan. 28	0.71
7	July 18	0.98
414	Aug. 7	1.29
7	" 29	1.26
414	Sept. 10	1.31
33	" 21	1.23
33	July 29	0.94
Mixed sample from several cows.	Oct. 14	1.22

TABLE III.

Utilization of Tryptophane by Mammary Gland.

Cow 7; lactating; July 18, 1925.

Sample No.	Tryptophane in blood plasma.	
	Jugular vein.	Mammary vein.
	mg. per 100 cc.	*mg. per 100 cc.*
1	0.97	0.84
2	0.99	0.79
3	1.00	0.81
4	0.97	0.81
Average.	0.98	0.81

Extreme difference between duplicate jugular vein samples.. 3 per cent.
" " " " mammary " " .. 6 " "
Difference between jugular and mammary vein averages..... 17 " "
Milk yield 14.1 kilos per day.

There was no unusual disturbance in taking samples of blood. The cannula was readily inserted into each vein and a good flow of blood obtained without difficulty. The collection of the samples of blood was started simultaneously; but, as the rate of flow was more rapid from the mammary vein, the collection was discontinued from 30 to 45 seconds sooner than from the jugular vein. 600 cc. of blood were taken from each vein. 5 minutes intervened from the time the halter was put on until the collection of blood was complete.

In Table II are given the results obtained from blood plasma. The concentration of the free tryptophane varies from 0.71 mg. to 1.31 mg. per 100 cc. with an average of 1.12 mg. as in the case of the whole blood.

Tables III to VIII show the tryptophane content of the plasma of blood taken simultaneously from the jugular and abdominal sub-

TABLE IV.

Utilization of Tryptophane and Amino N by Mammary Gland.

Cow 7; lactating; Aug. 28, 1925.

Sample No.	Tryptophane in blood plasma.		Amino N in blood plasma.	
	Jugular vein.	Mammary vein.	Jugular vein.	Mammary vein.
	mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
1	1.24	1.02	3.76	2.98
2	1.27	1.06		
Average.	1.255	1.04		

Difference between duplicate jugular vein tryptophane samples........ 2.4 per cent.
Difference between duplicate mammary vein tryptophane samples........ 4.0 " "
Difference between jugular and mammary vein tryptophane averages........ 17.1 " "
Difference between jugular and mammary vein amino N... 20.7 " "
Milk yield, 12.7 kilos per day.

This cow was brought into the stall at 9.35 a.m. and fed some alfalfa. At 10.50 the halter and antikicking device were placed on the cow. At 10.56 the cannula was inserted without difficulty into the jugular vein, and likewise at 10.57 into the mammary vein. The flow of blood started at 10.58 and continued 1 minute from the mammary vein and 1 minute and 45 seconds from the jugular vein. 740 cc. of blood were taken from each.

cutaneous (mammary) veins of lactating and non-lactating cows. In the two experiments with a lactating cow (Cow 7, Tables III and IV) the concentration of tryptophane in the plasma from the mammary vein was about 17 per cent lower than in that from the jugular vein. With the non-lactating animals (Cow 414, Tables V and VII, Cow 33, Tables VI and VIII) in which the flow of blood through the gland was very much less, these

differences were respectively 0.7, 8.4, 2.4, and 8.6 per cent, showing clearly a very much greater utilization of the free tryptophane in the blood plasma by the mammary gland during the process of milk secretion.

The experiment with Cow 7, Table IV (lactating), in which the free amino N, as well as the free tryptophane, was determined in the blood plasma from the jugular and mammary veins, shows that changes that were proportionately very nearly the same

TABLE V.

Utilization of Tryptophane by Mammary Gland.

Cow 414; non-lactating; Aug. 7, 1925.

Sample No.	Tryptophane in blood plasma.	
	Jugular vein.	Mammary vein.
	mg. per 100 cc.	*mg. per 100 cc.*
1	1.31	1.26
2	1.29	1.31
3	1.27	1.26
Average.	1.29	1.28

Extreme difference between duplicate jugular vein samples.. 3.1 per cent.
" " " " mammary " " .. 3.8 " "
Difference between jugular and mammary vein averages...... 0.7 " "

This cow was brought into the stall about 9.45 a.m. The halter and anti-kicking device were put on at 9.57½ a.m. The jugular cannula was inserted without difficulty at 9.58 and the mammary cannula likewise at 9.59¼. The flow of blood started at 9.59¾ and was stopped in both cases at 10.01. Only 3½ minutes, therefore, intervened between the placing of the halter on this cow and the end of the flow of blood. About 500 cc. were taken from each vein.

occurred in both of these blood constituents. The free tryptophane in the blood plasma from the mammary vein was 17.1 per cent lower than in the jugular vein, while this difference with the free amino N was 20.7 per cent. Cary (5) calculated that changes of this magnitude in the concentration of free amino N in the plasma of the blood of milking cows are approximately sufficient to account for the amino acids required in the synthesis of the proteins of milk. It follows, therefore, that the changes here

noted in the concentration of free tryptophane in the plasma of the blood when passing through the mammary gland are likewise of sufficient magnitude to account approximately for the quantity of this amino acid required in this process.

In neither experiment in which the concentration of the amino N in the plasma of blood from the jugular and mammary veins of non-lactating cows was determined, was that in the mammary

TABLE VI.

Utilization of Tryptophane and Amino N by Mammary Gland.

Cow 33; non-lactating; Sept. 21, 1925.

Sample No.	Tryptophane in blood plasma.		Amino N in blood plasma.	
	Jugular vein.	Mammary vein.	Jugular vein.	Mammary vein.
	mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
1	1.23	1.19	2.68	2.69
2	1.23	1.21		
Average.	1.23	1.20		

Difference between duplicate jugular vein tryptophane samples.......... 0.0 per cent.
Difference between duplicate mammary vein tryptophane samples.......... 1.7 " "
Difference between jugular and mammary vein tryptophane averages.......... 2.4 " "

There was no unusual delay or disturbance in taking these samples of blood. The halter and antikicking device were put on at 9.33½ a.m., jugular cannula inserted at 9.37; mammary cannula at 9.38; and the collection of blood started at 9.39. 2 minutes were required to collect the jugular vein sample and 1½ minutes to collect the mammary vein sample. 650 cc. of blood were taken from each vein.

vein lower than that in the jugular vein. This accords with previous work (5) along this line.

Attention should be drawn to the fact that in these experiments the tryptophane results with non-lactating cows seem to have been influenced by the conditions under which the samples were taken. Tables V and VI show the results when these samples were readily obtained and the cows were not unusually disturbed by the process; whereas Tables VII and VIII show the data with the same animals when the contrary conditions prevailed. With the former

(Cow 414, Table V, and Cow 33, Table VI) the concentration of the free tryptophane in the mammary vein blood plasma was 0.7 and 2.4 per cent less than in the jugular vein; whereas with the latter (Cow 414, Table VII, and Cow 33, Table VIII) these differences are 8.4 and 8.6 per cent. We have no satisfactory explana-

TABLE VII.

Utilization of Tryptophane and Amino N by Mammary Gland.

Cow 414; non-lactating; Sept. 10, 1925.

Sample No.	Tryptophane in blood plasma.		Amino N in blood plasma.	
	Jugular vein.	Mammary vein.	Jugular vein.	Mammary vein.
	mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
1	1.29	1.19	2.97	3.16
2	1.33	1.21		
Average.	1.31	1.20		

Difference between duplicate jugular vein tryptophane samples.. 3.0 per cent.
Difference between duplicate mammary vein tryptophane samples.. 1.7 " "
Difference between jugular and mammary vein tryptophane averages.. 8.4 " "
Difference between jugular and mammary vein amino N.... 6.0 " "

This cow was considerably disturbed while the sample of blood was taken from the jugular vein. It had been kept in the stall when the other cows were let out in the morning. At 10.50 a.m. the halter and antikicking arrangement were placed on it. At 10.55 the cannula was inserted into the skin to try for the left jugular vein. After several unsuccessful attempts it was withdrawn, and finally a flow of blood was obtained from the right jugular vein. The mammary cannula was inserted at 11.01½ a.m., and the flow of blood started at 11.03½. The mammary vein collection was complete at 11.05½, and the jugular at 11.09½, 650 cc. of blood being taken from each vein.

tion of how the difference in the conditions under which these samples of blood were taken affected the results. These cows had previously given milk, and it was possible at the time to obtain milk from their udders. It may be that synthetic processes of this sort, with corresponding autolyses, are proceeding constantly to

some extent during this period. In one of these experiments the difficulty encountered was in obtaining the blood from the jugular vein, whereas in the other it occurred in taking the mammary vein sample. In either instance, however, the udder may have been greatly disturbed, the muscles surrounding it contracted, and

TABLE VIII.

Utilization of Tryptophane by Mammary Gland.

Cow 33; non-lactating; July 29, 1925.

Sample No.	Tryptophane in blood plasma.	
	Jugular vein.	Mammary vein.
	mg. per 100 cc.	*mg. per 100 cc.*
1	0.89	0.88
2	0.97	0.83
3	0.93	
4	0.95	
Average.	0.93	0.85

Extreme difference with duplicate jugular vein samples....... 6.3 per cent.
Difference between duplicate mammary " " .... 5.7 " "
" " jugular and mammary vein averages.... 8.6 " "

It was very difficult to obtain a flow of blood from the mammary vein of this cow. The halter and antikicking device were put on at 9.55½ a.m. The cannulæ were inserted and the flow of blood from both veins started at 9.58¾. The collection of jugular blood was complete at 10.00½. The flow of mammary blood, however, was very slow, and the udder and vein were disturbed in an effort to promote it. The last of this blood sample came in drops from the cannula. Practically all of the blood that was obtained from this vein was collected by 10.04½. It was 210 cc. The jugular sample was 550 cc.

blood thereby mechanically forced back into circulation. This blood may have been deprived more completely of the materials used in the synthetic processes or have contained less of the products of autolysis than is usual with blood leaving the gland at this time. This explanation, however, is not consistent with the amino N results, which, as noted above, were not lower in the mammary vein than in the jugular vein blood.

SUMMARY.

Data are given on the free tryptophane in cow blood and blood plasma under average conditions of feeding. In the blood it varied from approximately 1.0 to 1.5 mg. per 100 cc. with an average of 1.12 mg. In the blood plasma it varied from 0.71 to 1.31 mg. per 100 cc. with an average of 1.12 mg. also.

Data are given to show that with lactating cows the concentration of free tryptophane in blood plasma is lower in the abdominal subcutaneous (mammary) vein than in the jugular vein. The average difference was about 17 per cent. With non-lactating cows the results varied according to the disturbance of the animal when the samples of blood were taken. When the blood was readily obtained and the cow was not greatly disturbed, the differences between the concentration of free tryptophane in jugular and mammary vein blood plasma were practically negligible—0.7 and 2.4 per cent. When the contrary conditions prevailed, these differences were 8.4 and 8.6 per cent in two different experiments.

Since there is a very much greater circulation of blood through the mammary glands during lactation, these figures are interpreted as demonstrating the utilization of the free tryptophane in blood plasma in milk secretion.

Determinations were made of the concentration of amino N in the plasma from these samples of jugular and mammary vein blood. With lactating cows the differences were proportionately very nearly the same as the changes observed in the concentration of free tryptophane.

The differences observed in the concentration of free tryptophane in jugular and mammary vein blood plasma are adequate to account for the amount of this amino acid required in the synthesis of the milk proteins.

BIBLIOGRAPHY.

1. Cary, C. A., and Meigs, E. B., *J. Agric. Research*, 1924, xxix, 603.
2. Cary, C. A., *J. Biol. Chem.*, 1928, lxxviii, 377.
3. Kaufmann, M., and Magne, H., *Compt. rend. Acad.*, 1906, cxliii, 779.
4. Meigs, E. B., Blatherwick, N. R., and Cary, C. A., *J. Biol. Chem.*, 1919, xxxvii, 1.
5. Cary, C. A., *J. Biol. Chem.*, 1920, xliii, 477.
6. Meigs, E. B., *Physiol. Rev.*, 1922, ii, 204.

NOTE ON THE NITROGEN EXCRETION OF CAMELS.*

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Read[1] has reported the nitrogen distribution in the urine of the Bactrian camel (pregnant female). Read's data show the unusual circumstance that urea and ammonia are lacking from the urine of this animal.

We have recently had the opportunity to examine urine from members of each species of the camel family, and do not confirm Read's observation but find that the Bactrian camel, as well as the dromedary, the llama, and the alpaca, excretes on an average 60 per cent of its total nitrogen as urea.

The urine samples were collected by the keeper at the New York Zoological Park.[2] About 100 cc. of urine were placed in a bottle containing 15 to 25 cc. of 20 per cent trichloroacetic acid and mailed to Charlottesville for analysis. The exact dilution of the urine by the acid was not noted because we were not interested in the absolute nitrogen content. We have found that this method of preservation effectively checks all decomposition of urea to ammonia, and we believe that within a period of 48 hours—the usual time intervening before analysis—the dehydration of creatine to creatinine is negligibly small. We do not, however, place emphasis on the creatine-creatinine distribution.

Urinalysis was carried out by the usual methods; total nitrogen by Folin's micro-Kjeldahl method and direct Nesslerization; urea by Folin and Youngburg's urease method, after extraction of the ammonia with permutit; ammonia by Folin and Bell's permutit

* These observations were made during the course of other work supported by a grant from the Elizabeth Thompson Science Fund.

[1] Read, B. E., *J. Biol. Chem.*, 1925, lxiv, 615.

[2] We are indebted to Professor R. L. Ditmars for permission to obtain these urine samples.

method; uric acid by Benedict and Franke's method; amino acids and creatinine by Folin's method; and creatine by Folin's water bath procedure.

The first series of data in Table I is quoted from Read, and represents the analysis of a 24 hour specimen, the average of "1 month's estimations conducted under standard dietary conditions." Following these are our data on individual samples of urine.

TABLE I.

The results are expressed in per cent of total N.

	Total N.	Urea N.	Uric acid N.	Ammonia N.	Amino acid N.	Creatinine N.	Creatine N.	Hippuric acid N.	Purine N.	Rest N.
	mg. per 100 cc.									
Camelus bactrianus, ♀, pregnant	*	Trace.				24.8	14.6	35.0	19.9	5.7
Camelus bactrianus, ♀, pregnant.										
Mar. 12	1460	68.5	1.2	1.7	1.2	7.3	3.8			16.1
" 22	1617	69.0	0.8	1.7	1.9	10.5	1.7			14.5
" 26	814	65.2	1.8	3.8	2.1	12.4	0.7			14.0
" 29		Gave birth to female.								
Apr. 8	1960	62.7	1.0	4.1	1.8	10.8	6.9			12.7
Camelus dromedarius, ♂.										
Feb. 25	2700	55.5	0.3	12.3	1.7	14.4	3.2			12.7
Mar. 19	2405	32.5	0.3	19.1	0.2	13.6	4.5			19.8
Auchenia huanacos, ♂.										
Feb. 17	640	67.7	0.8	2.2	1.3	6.1	3.0			19.0
Mar. 9	1820	60.5	0.7	1.8	1.4	10.2	1.5			23.9
Auchenia vicunna, ♂.										
Feb. 25	1768	59.6	0.3	4.5		4.6	2.7			28.3

* 24 hour sample; total nitrogen 8.70 gm.

It may be noted that Read's camel was pregnant; we had the good fortune to obtain urine from a pregnant Bactrian camel, and again after parturition.

The discrepancy between Read's data and ours is difficult to explain, especially since we both account for the greater fraction of the nitrogen. It is hard to believe, though perhaps possible,

that a difference in dietary régime can cause the total disappearance of urea from the urine of a mammal. Read states that his camel was fed on sorghum leaves, sweet potato vines, and salt; ours was fed on oats and timothy hay. Both presumably had water *ad libitum*.

In conflict with Read, we find ammonia to be present, but we cannot guarantee that this ammonia was not formed before the addition of the trichloroacetic acid. If it was originally present, the association of ammonia with urea in our camel, and the total absence of both of these substances in the case of Read's camel, may be significant.

SUMMARY.

In contradiction of Read's observation that the Bactrian camel excretes no urea, we find that this animal, as well as the other members of the camel family, excretes urea in amounts comparable with other herbivorous mammals.

CHANGES IN THE OXYGEN CAPACITY OF THE BLOOD PIGMENT OF RABBITS FOLLOWING PARTIAL HEPATECTOMY.

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INTRODUCTION.

In a preceding experiment (1) it was found that in ten splenectomized rabbits a non-oxygen-carrying hemoglobin appeared in the circulating blood after operation but disappeared again in from 3 to 6 days. It seemed possible that the removal of some other organ might cause similar changes in the blood pigment and we decided to study the effect of partial removal of the liver on the oxygen capacity of rabbit blood.

Methods.

From 75 to 80 per cent of the liver was removed, the small posterior and the Spigelian lobes being left in place. Ether anesthesia was used in every instance and the operation performed as rapidly and bloodlessly as possible.

When possible, blood samples, from 3 to 5 cc. in amount, were taken from the ear veins but in most cases cardiopuncture was necessary after the operation. It was found that the method of obtaining the blood did not affect the results. All blood samples were oxalated. Initial bleedings were made 2 or 3 days before the operation and further specimens were obtained from 1 to 9 hours after. The total blood pigment and the oxygen capacity of each blood sample was determined by the Van Slyke carbon monoxide method (3), 0.2 cc. of blood being used for each analysis. All samples were saturated with New York City illuminating gas and all determinations were checked within 0.2 volume per cent.

Spectrophotometric readings were also made on each blood sample, with a technique similar to that used in preceding investigations (1, 2).

Results.

In six rabbits about three-quarters of the liver was removed. Two of these animals died within 2 hours of the operation from hemorrhage and, possibly, liver insufficiency. All six were given 5 per cent glucose solution subcutaneously and to drink. In three control animals the peritoneal cavity was opened under

TABLE I.

Amount of Non-Oxygen-Bearing Hemoglobin in Per Cent of Total Blood Pigment.

The last three experiments are the controls.

Rabbit No.	Initial.	Hours after operation.									
		½	1	2	3	4	5	6	7	9	26
127	0	1.79*									
51	0			1.9*							
17	0				9.95		5.39		0		0
111	0		0			11.85			0		
68	0					4.36		5.45		0	
204	0			0		5.78*					
13	0				0		0				
145†	0			0							
199	0					0					

* Died before further samples could be obtained.

† Hemorrhage from the femoral vein.

ether anesthesia but the liver left untouched. In one of these about 30 cc. of blood were drawn from the femoral vein to see whether or not the factor of blood loss played any part in the results.

A summary of the data is given in Table I. In the initial sample before operation in every instance the total blood pigment and the oxygen-carrying hemoglobin agree within experimental error. In the three control animals this same agreement persists after the operation. In each of the six partially hepatectomized rabbits, however, there appears after the operation a discrepancy

between the total pigment and the oxygen-carrying compound. This occurs in the 3rd and 4th hours most markedly (earlier in the two cases that did not survive more than 2 hours) and disappears in the 8th hour.

Coincident with the appearance of this discrepancy occur changes in the spectrophotometric readings. The ratios obtained for the light absorption of the initial blood samples at two given wave-lengths (Å. = 5400 and 5600) are around 1.60, suggestive of the characteristic value for oxyhemoglobin. Following the operative procedure the ratios increase to 1.80 and above in the 3rd and 4th hours and return to the lower values by the 9th hour. No such changes are found in the blood of the control animals. No ratios suggestive of methemoglobin were obtained. The variations in the spectrophotometric readings are similar to those observed in the blood of rabbits following splenectomy (1).

DISCUSSION.

From the foregoing data it would seem that the removal of three-quarters of the liver of a rabbit produces a change in the hemoglobin similar to that caused by the removal of the spleen. In the former case the change occurs sooner and is more transient. In both instances there appears a difference between the total pigment and the oxygen-carrying hemoglobin, possibly due to the formation of a non-oxygen-bearing hemoglobin derivative which resembles methemoglobin but seems to have a characteristic spectrophotometric ratio of its own. To questions concerning the nature and significance of this derivative we are as yet unable to suggest an answer.

The operative technique used in the experiment is that adopted by Dr. McMaster and his associates at the Rockefeller Institute to whom the writers wish to make grateful acknowledgment.

BIBLIOGRAPHY.

1. Stimson, B. B., *J. Biol. Chem.*, 1927, lxxv, 95.
2. Stimson, B. B., *J. Biol. Chem.*, 1927, lxxv, 741.
3. Van Slyke, D. D., *J. Biol. Chem.*, 1925, lxvi, 409.

FURTHER INVESTIGATIONS CONCERNING THE SPECIFIC ACTION OF SALTS IN THE EXTRACTION OF UREASE FROM AMEBOCYTES OF LIMULUS.

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In a previous communication, Loeb and Bodansky[1] have shown that extracts of urease from amebocyte tissue of *Limulus*, prepared with various salts, differ very markedly in their activity, and that the salts of the alkaline earths are especially favorable. We considered at that time the possibility that these differences in salt action may be due to differences not in the extracting power but in the specific action of the salts on the enzyme. During the past summer we began therefore an analysis of the salt action on urease extracted from the amebocyte tissue.

Method of Preparing Extracts and of Testing Their Strength.—We introduced certain modifications in the technique used by us previously.[2] In order to make our various experiments comparable we standardized the quantities of material used and the length of the period during which they acted. In each case we added to 1.5 gm. of amebocyte tissue 0.5 cc. of toluene and 1 gm. of sea-sand and ground this mixture thoroughly for 1.5 minutes; 40 cc. of the extractive were then added and thoroughly mixed. The period of extraction was 2 hours at room temperature. We then filtered the mixture and to 25 cc. of the filtrate added 25 cc. of 1 per cent urea solution, and (except in the earlier experiments) 0.5 cc. of toluene. This mixture was allowed to stand 22 hours at 30°. At the end of this period we filtered and used 25 cc. of the filtrate for ammonia determination, which was carried out as previously

[1] Loeb, L., and Bodansky, O., *J. Biol. Chem.*, 1927, lxxii, 415.

[2] Loeb, L., and Bodansky, O., *J. Biol. Chem.*, 1926, lxvii, 79.

reported, except that besides the 5 drops of caprylic alcohol, 25 cc. of a saturated solution of potassium carbonate were quickly added instead of the 10 gm. of sodium carbonate. The number of cc. of 0.04 M HCl which had been neutralized by the ammonia driven over by aeration (lasting 2 to 3 hours) was multiplied by 2, because only half the amount of the incubation mixture had been used for aeration. By subtracting from this figure of neutralized 0.04 M HCl, the amount of 0.04 M HCl neutralized in the blank experiment, we obtained the coefficient of efficiency of the urease in the various mixtures. The efficiency coefficients alone will be given in our tables.

We found the second addition of toluene to the mixture of extract and urea solution to be necessary, if we wished to insure the absence of microorganisms. With this addition bacterial action was excluded in the large majority of experiments. However, when bacteria did develop they did not produce ammonia to any noticeable extent and did not therefore markedly influence the results.

The activity coefficients given in our tables are usually the means of the figures obtained in several experiments; the number of individual determinations in each experiment is the figure added in parentheses after the coefficients.

Efficiency Coefficients of Extracts Made with Salts and Certain Colloids.—Water is a very inefficient extractive. Sumner[3] observed that water very rapidly inactivated the "crystalline" urease prepared by him from jack bean. He concludes that traces of lead admixed to the water distilled through a metal condenser are responsible for the destructive action of H_2O in his experiments. In redistilling water we used Pyrex glass. It is, therefore, improbable that the inefficiency of water in our experiments is due to the admixture of heavy metals. On the other hand, colloids like gum arabic prevented the inactivating effect of water in Sumner's experiments. Extracts of Na and K chlorides and sulfates showed only very weak activity in the preparation of urease from amebocyte tissue, although they are probably somewhat more efficient than water. $AlCl_3$ and $La(NO)_3$ extracts are almost inactive; also the sodium citrate extract is very weak

[3] Sumner, J. B., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 287. Sumner, J. B., and Hand, D. B., *J. Biol. Chem.*, 1928, lxxvi, 149.

(Table I). Therefore, salts with trivalent cations or anions showed little activity in our experiments.

In contrast with these weak extracts is the very marked activity of the chlorides of the alkaline earths. Again $MgCl_2$ stands midway between the salts of the alkaline earths and the salts of Na and K. $MnCl_2$ is, as mentioned before, much more active than

TABLE I.

Efficiency Coefficients in Cc. of 0.04 M HCl.

Extractive.	Efficiency coefficient.	
H_2O	0.48	(2)*
0.50 M NaCl	2	(15)
0.50 " KCl	2	(2)
0.38 " $CaCl_2$	130	(18)
0.38 " $SrCl_2$	102	(4)
0.38 " $BaCl_2$	80	(4)
0.38 " $MgCl_2$	37	(12)
0.38 " $MnCl_2$	10	(3)
0.38 " $CuCl_2$	2	(3)
0.38 " $ZnCl_2$	0.66	(3)
0.30 " $FeCl_3$	2	(2)
0.30 " $AlCl_3$	0.00	(2)
0.30 " $La(NO)_3$	0.44	(2)
0.38 " Na_2SO_4	2	(2)
0.38 " K_2SO_4	2	(2)
0.30 " Na_3 citrate	1	(2)
0.5 " NaCl in:		
1 per cent gelatin solution	4	(2)
2 " " gum arabic solution	5	(2)
2 ' " boiled starch	1	(1)
Sea water	4	(1)

* Throughout the tables the numbers in parentheses indicate the number of individual determinations included in the experiment.

the salts of the heavy metals, which are only very slightly effective. However, the average activity of $MnCl_2$ was, in our recent experiments, somewhat less than in our former experiments.

Sea water is not very effective as an extractive, while unheated *Limulus* serum, which contains about the same kinds of salts as sea water, although in somewhat different proportions, is very active as we shall see later. This difference in the activity of these

two solutions must be due to the protective effect of the protein which is present in the unheated *Limulus* serum. Also, *Limulus* serum heated to 80° is more effective than sea water or 0.5 M NaCl solution. While thus the protein of *Limulus* serum greatly improves the action of the salt mixture, the addition of other colloids,

TABLE II.

*Effect of Varying Concentration of Extracting Salt Solutions.**

Extractive.	Average efficiency coefficients.	pH after urease action.
0.2 M NaCl	0.78 (2)	6.4
0.5 " "	2 (4)	7.3
1.0 " "	4 (2)	8.9
1.25 " "	11 (4)	9.2
1.5 " "	15 (2)	9.1
1.75 " "	8 (2)	9.1
2.0 " "	21 (4)	9.2
0.1 " $CaCl_2$	1 (2)	6.1
0.25 " "	47 (3)	8.2
0.38 " "	114 (3)	7.5
0.75 " "	100 (3)	7.3
1.0 " "	112 (2)	7.4
1.25 " "	84 (2)	7.1
1.5 " "	61 (2)	7.1
2.0 " "	6 (4)	8.6
0.05 " $MgCl_2$	0.52 (1)	6.6
0.1 " "	1 (1)	6.6
0.19 " "	6 (2)	7.9
0.38 " "	36 (4)	8.8
0.75 " "	33 (2)	8.5
1.0 " "	26 (3)	8.4
1.50 " "	19 (1)	8.6
2.0 " "	10 (1)	8.2

* This table does not contain all our determinations of the activity of these substances, but only those made for these particular experiments.

like gelatin, gum arabic, and boiled starch to either 0.5 M NaCl or water, does not, or only very slightly, exert a beneficial effect.

Effect of Concentration of Salts and Non-Electrolytes on Activity of Extracts.—The optimal concentration of NaCl is in the range between 1.25 M and 2.0 M; that of $CaCl_2$ and $MgCl_2$ between 0.38 M and 1.0 M (Table II). The curves rise very steeply to the opti-

mal point with $CaCl_2$, somewhat less so with $MgCl_2$, and still less with NaCl. When higher concentrations are used, the curve falls again with $CaCl_2$ and $MgCl_2$, but less abruptly than it rose. In the case of NaCl the descending branch of the curve has not as yet been observed.

As to the changes in the pH in the mixture of extract and urea solution at the end of the experiment, we find in the NaCl extract a rise in the pH to about 9.0 as soon as the efficiency coefficient reaches 4 to 5. Then it remains at 9 or rises slightly. In the case of the $CaCl_2$ extract the pH always remains below 9, although the efficiency coefficient is in this case much higher than in the case of NaCl. With a 0.25 M $CaCl_2$ extract, the coefficient is 47 and the pH 8.2; then it falls and remains between 7.0 and 7.5, until a 2.0 M concentration of $CaCl_2$ is reached, when it declines to

TABLE III.

Effect of Variations in Concentration of Glycerol Solutions on Efficiency Coefficient of Extracts.

Extractive.	Efficiency coefficient.
5 per cent glycerol	0.72 (1)
10 " " "	0.62 (2)
25 " " "	4 (2)
50 " " "	10 (1)

6 and the pH again rises to 8.6. With the fall of the coefficient the pH therefore rises.

In the case of the $MgCl_2$ extract, the pH rises to 9, when the activity coefficient reaches its maximum. At 0.19 M $MgCl_2$ there is a noticeable rise in some experiments. With a still greater concentration of $MgCl_2$ and a gradual, although slow decline in the activity, there seems to take place a moderate fall in the pH.

While in the case of $CaCl_2$, and also of $MgCl_2$, it is evident that we are dealing with specific salt or ion actions, the improvement which takes place in the NaCl extracts, with increasing concentration of the NaCl solution, is probably due not to the specific salt action, but to the increased osmotic pressure which has a preserving effect on the extracted substance. This conclusion is confirmed by an experiment in which we studied the effect of

variations in the concentrations of glycerol solutions on the efficiency coefficient of the extract.

Table III suggests that with the increase in concentration the effectiveness of a glycerol solution increases.

Optimal Concentration of Acid and Alkali in Preparation of Extract.—In Table IV our former results are included. The optimum for alkali action agrees approximately with our previous finding; it is situated at about 0.005 M NaOH, but the coefficients are still high at 0.002 M and also at 0.01 M NaOH. However, at the latter point they would have been lower, if it had not been for one experiment in which the period of extraction was prolonged

TABLE IV.

Optimal Concentration of Acid and Alkali in Preparation of Extract.

Extractive.	Efficiency coefficient.	pH after urease action.
0.5 M NaCl	1 (2)	
0.5 " " in 0.001 N NaOH	9 (3)	9.4
0.5 " " " 0.002 " "	23 (5)	9.1
0.5 " " " 0.005 " "	52 (2)	9.4
0.5 " " " 0.01 " "	25 (5)	9.5
0.5 " " " 0.0125 " "	20 (1)	
0.5 " " " 0.02 " "	2 (2)	10.0
0.5 " " " 0.05 " "	1	
0.5 " " " N/3000 HCl	36 (1)	9.5
0.5 " " " 0.0005 N "	34 (2)	9.6
0.5 " " " 0.001 " "	30 (3)	8.0
0.5 " " " 0.002 " "	6 (2)	8.8

and the coefficient very high. The coefficients decrease again at 0.01 M and 0.0125 M NaOH. In our tests concerning acid as extractive, the optimum had a range varying between M/3000 and 0.001 M HCl; it decreased rapidly with a still stronger concentration.

Comparison of Neutralizing Effects on Alkali of NaCl, $CaCl_2$, and $MgCl_2$ Extracts.—In our previous experiments we found that if we compare the effects on the pH of the addition of equal amounts of 0.01 M NaOH to extracts made with 0.5 M NaCl or with 0.38 M $CaCl_2$, the pH is higher after addition to the former. $CaCl_2$ extract has a stronger buffer action than NaCl extract. We obtained similar results in three additional experiments, in two of

which we used 10 cc. for each titration of the two extracts and in the third experiment 25 cc. However, the differences between the neutralizing power of the two kinds of extract were not equally marked in all experiments. $MgCl_2$ extract behaved in one experiment, when 10 cc. of the extract were used, in a manner similar to the $CaCl_2$ extract, whereas in a second experiment, when 25 cc. of the extract were used, it behaved in a way intermediate between the NaCl and $CaCl_2$ extracts. There is, therefore, in the $CaCl_2$ and also in the $MgCl_2$ extract, a greater amount of buffer substance present than in the NaCl extract and this substance may be at least partly responsible for the lower pH which is observed in the mixtures of the $CaCl_2$ extract with urea solutions, at the end of the incubation period. However, it is probable that the formation of $CaCO_3$ from $CaCl_2$ and $(NH_4)_2CO_3$ is the most important factor in the partial neutralization of the $CaCl_2$ extract.

Effect of Addition of $CaCl_2$ or of NaCl to Extract Made with 0.5 M NaCl on Efficiency Coefficient of Extract.—We have formerly seen that addition of an otherwise optimal amount of $CaCl_2$ to an extract made with 0.5 M NaCl does not produce an improvement in the activity of the extract. We repeated this experiment (Table V, Experiments A and B) and varied it by adding a larger amount of $CaCl_2$ to the extract corresponding to a concentration of 2 M $CaCl_2$, without producing an improvement (Table V, Experiment C). Also, we prepared the extract with 2.0 M NaCl instead of with 0.5 M NaCl, because we had previously found that the coefficient of the former is much greater than that of the latter. However the addition of an amount of $CaCl_2$ to the extract made with 2.0 M NaCl, sufficient to produce a concentration of 0.38 M $CaCl_2$ in the mixture, does not lead to an improvement in the efficiency of the extract (Table V, Experiments D and E). Similarly, if after extraction of amebocyte tissue with water, an amount of $CaCl_2$ sufficient to produce a 0.38 M concentration is added, no improvement in the activity is produced (Table V, Experiment G). These results confirm those previously obtained by Loeb and Bodansky.[1] If we first extract the amebocyte tissue with 0.5 M NaCl and afterwards add enough NaCl in substance to produce a concentration corresponding to 2.0 M NaCl in the extract, no improvement is produced and the coefficient of the mixture resembles that of a 0.5 M NaCl extract rather than that

of a 2.0 M NaCl solution (Table V, Experiment F). We may conclude from this experiment that a 0.5 M NaCl concentration of NaCl produces an injurious effect on the urease, and thus prevents a subsequent addition of $CaCl_2$ or of a higher concentration of NaCl from exerting a beneficial action.

Effect of Addition of $CaCl_2$ to Extract Made with 0.38 M $MgCl_2$ Solution on Efficiency Coefficient of Extract.—Different results were obtained, if, instead of adding $CaCl_2$ to extracts made with NaCl, we added it to an extract made with $MgCl_2$ solution. In this case an improvement was produced if the $CaCl_2$ was added after the extraction from the amebocyte tissue had been concluded. As in the former experiment, the $CaCl_2$ was added in substance

TABLE V.

Effect on Efficiency Coefficient of Extract of Addition of $CaCl_2$ or NaCl to Extract Made with NaCl.

Experiment.	Extractiva.	Substance added to extract in quantity sufficient to produce stated concentration in extract.	Efficiency coefficient.
A	0.5 M NaCl.		1.35 (3)
B	0.5 " "	0.38 M $CaCl_2$.	0.47 (2)
C	0.5 " "	2.0 " "	1 (1)
D	2.0 " "		26 (2)
E	2.0 " "	0.38 M $CaCl_2$.	29 (2)
F	0.5 " "	1.5 " NaCl (altogether 2.0 M NaCl).	1.80 (1)
G	H_2O.	0.38 " $CaCl_2$.	0.08 (1)

to the extract in such a quantity that it was present in definite concentrations.

We see (Table VI, Experiments A, B, and C) that the subsequent addition of $CaCl_2$ (0.38 M) to the extract made with 0.38 M $MgCl_2$ produces an activity (109) which is almost exactly intermediate between that of 0.38 M $MgCl_2$ and of 0.38 M $CaCl_2$ extracts. The theoretically required figure is 109. The figures in Experiments A, B, and C represent the means of five experiments. Also the pH of the mixture is intermediate. In Experiment C the osmotic pressure of the mixture is increased over that in Experiments A and B; but according to the findings in Table II this increase should not affect the result in an unfavorable manner. These

results can be interpreted as indicating that the enzyme in combination with Mg was protected and subsequently either directly or indirectly combined in equal amounts with $MgCl_2$ and $CaCl_2$.

In Experiment E, where to an extract, made with a less concentrated solution of $MgCl_2$, a relatively small amount of $CaCl_2$ has been added, but an amount sufficient to raise the osmotic pressure of the mixture to approximately that of a 0.38 M $CaCl_2$ solution, the efficiency coefficient is distinctly increased, although it still remains considerably below that of 0.38 M $CaCl_2$ solution or even below that of the mixture of Experiment C. In Experiment F a 0.19 M $MgCl_2$ solution has a coefficient of 5.50, which is approximately the same as that given in Table II. Addition of enough

TABLE VI.

Effect on Efficiency Coefficient of Extract of Addition of $CaCl_2$ to Extract Made with $MgCl_2$.

Experiment.	Extractive.	Substance added to extract in quantity sufficient to produce stated concentration in extract.	Efficiency coefficient.	pH after urease action
A	0.38 M $MgCl_2$.		47 (5)	9.0 (5)
B	0.38 " $CaCl_2$.		171 (5)	7.4 (5)
C	0.38 " $MgCl_2$.	0.38 M $CaCl_2$.	109 (5)	8.2 (5)
D	0.285 " "		34 (2)	8.9 (2)
E	0.285 " "	0.095 M $CaCl_2$.	62 (3)	8.7 (3)
F	0.19 " "		5.50 (2)	8.6 (2)
G	0.19 " "	0.19 M $CaCl_2$.	9 (3)	8.7 (3)

$CaCl_2$ to produce a concentration equal to 0.19 M $CaCl_2$, increases the coefficient somewhat, probably to a degree corresponding to the amount of $CaCl_2$ added.

Extraction of Urease by Mixtures of NaCl and $CaCl_2$ or of $MgCl_2$ and $CaCl_2$.—In the following experiments, instead of adding $CaCl_2$ to the extract we used, as extractives, mixtures of NaCl and $CaCl_2$ or of $MgCl_2$ and $CaCl_2$.

A comparison of Table VII (Experiments A to G) with Table V shows clearly the difference in the results obtained in case $CaCl_2$ is added to a NaCl solution before and after extraction. If added after extraction, $CaCl_2$ is ineffective. If added before extraction to a 0.5 M NaCl solution, it is active; but the activity

is less than when a pure $CaCl_2$ extract is used. The inhibiting effect of NaCl seems to be greater if a 2.0 M NaCl solution is used, although the latter, if used alone as an extractive, preserves the urease better than a 0.5 M NaCl solution. A comparison of Table VII (Experiments H to L) with Table VI shows that $CaCl_2$ is effective when it is added to $MgCl_2$ solutions before as well as

TABLE VII.

Extraction of Urease by Mixtures of NaCl and $CaCl_2$ and of $MgCl_2$ and $CaCl_2$.

Experiment.	Extractive.	Efficiency coefficient.			pH after urease action.
A	0.5 M NaCl	2.60	(3)		7.45
B	0.38 " $CaCl_2$	161	(3)		7.45
C	0.5 " with respect to NaCl, 0.38 M with respect to $CaCl_2$	119	(3)		7.45
D	2.0 M NaCl	17	(1)		9.0
E	0.38 " $CaCl_2$	140	(1)		7.2
F	Solution 2.0 M with respect to NaCl, 0.38 M with respect to $CaCl_2$	62	(1)		7.8
G	Solution 1.0 M with respect to NaCl, 0.19 M with respect to $CaCl_2$	36	(1)		8.4
H	0.38 M $MgCl_2$	41	(4)		8.95
I	0.38 " $CaCl_2$	145	(4)		7.5
L	Solution 0.38 M with respect to both $MgCl_2$ and $CaCl_2$	112	(4)		7.85
			Exp. I.	Exp. II.	
M	0.38 M $MgCl_2$	36	49	23	9.2
N	0.38 " $CaCl_2$	178	191	164	7.5
O	30 cc. 0.38 M $MgCl_2$ + 10 cc. 0.38 M $CaCl_2$	57	84	30	8.9
P	20 cc. 0.38 M $MgCl_2$ + 20 cc. 0.38 M $CaCl_2$	83	128	37	8.2
Q	10 cc. 0.38 M $MgCl_2$, 30 cc. 0.38 M $CaCl_2$	90	139	40	7.8

after extraction. Table VI, Experiment G, suggests that the activity of the mixture may perhaps be somewhat greater when $CaCl_2$ is added to $MgCl_2$ before extraction. However, a comparison of Table VI, Experiment C, with Table VII, Experiment C indicates that, if such a difference does exist, it cannot be great. Table VII (Experiments M to Q) shows that in the mixture of

$MgCl_2$ and $CaCl_2$ the activity is the higher, the greater the amount of $CaCl_2$ admixed to the extracting solution. This effect comes out more clearly in Experiment I, where the activity of the extract is greater, than in Experiment II, although the results of the latter also agree with this conclusion.

These findings would be in harmony with the assumption that the enzyme, or a substance markedly influencing its activity, is combined with various cations and that the efficiency coefficients of the urease in these combinations differ greatly. The Mg combination is not so potent as the combinations with Ca, Ba, or Sr, but the Mg combination preserves the enzyme in such a condition that afterwards Ca can take the place of Mg. In a mixture of Ca and Mg salts both Mg and Ca combinations apparently form side by side and the coefficient is therefore intermediate. On the other hand, the combinations with the alkali metals seem to be of such a nature that the enzyme is changed irreversibly and subsequently an effective combination with Mg or Ca cannot be produced. It is therefore necessary to add the salt of the alkali earth to the salt of the alkali metal in the beginning of the extraction, if the activity coefficient is to be raised above that of NaCl extract. The pH of the mixtures of extract and urea solution at the end of the experiment is low in the case of the NaCl extract (Table VII, Experiment A), because the amount of $(NH_4)_2CO_3$ formed is, in this case, very small. The pH is 9.0 in the mixture with hypertonic NaCl solutions in which the amount of $(NH_4)_2$-CO_3 formed is greater. It is similar with 0.38 M $MgCl_2$; but with $CaCl_2$ solutions it is between 7 and 7.5, although in this case the amount of $(NH_4)_2CO_3$ produced is very high. It is somewhere intermediate between 9.0 and 7.0 with mixtures of $CaCl_2$ and hypertonic NaCl and of $CaCl_2$ and $MgCl_2$ solutions.

Effect of Addition of NaCl and $MgCl_2$ and Salts of Heavy Metals to Extracts Prepared with $CaCl_2$.—In the experiments recorded so far, we tested the effect of the addition of $CaCl_2$ to extracts made with NaCl or with $MgCl_2$ solutions. In the following experiments we used 0.38 M $CaCl_2$ solutions as extractives and tested the effect of the subsequent addition of NaCl, $MgCl_2$, or of the salts of heavy metals to the extracts; we also added similar salts to extracts made with 0.38 M $BaCl_2$ or $SrCl_2$ instead of with $CaCl_2$.

Table VIII should be compared with Tables V, VI, and VII.

Whereas Table V shows that, if $CaCl_2$ is added to NaCl extracts, no improvement is produced in the urease action, Table VIII (Experiments A, B, and C) shows that, if NaCl is added to $CaCl_2$ extracts, the coefficients of the mixtures are definitely lower than without subsequent addition of NaCl, and the lowering is the greater the stronger the concentration of NaCl. The results are somewhat similar to those in Table VII, where extracts made with mixtures of 0.5 M NaCl and 0.38 M $CaCl_2$ likewise show lower co-

TABLE VIII.

Effect of Addition of NaCl, $MgCl_2$, and Salts of Heavy Metals to Extracts Prepared with $CaCl_2$, $BaCl_2$, or $SrCl_2$.

Experiment.	Extractive.	Substance added to extract in quantity sufficient to produce stated concentration in extract.	Efficiency coefficient.
A	0.38 M $CaCl_2$.		131 (7)
B	0.38 " "	0.5 M NaCl.	56 (5)
C	0.38 " "	2.0 " "	29 (1)
D	0.38 " "	0.38 " $CuCl_2$.	0.72 (1)
E	0.38 " "	0.30 " $FeCl_3$.	0.48 (1)
F	0.285 " "		164 (1)
G	0.285 " "	0.095 M $MgCl_2$.	108 (1)*
H	0.38 " $BaCl_2$.		119 (2)
I	0.38 " "	0.5 M NaCl.	69 (2)
L	0.38 " "	0.38 " $ZnCl_2$.	0.40 (1)
M	0.38 " "	0.38 " $CuCl_2$.	2 (1)
N	0.38 " "	0.38 " $FeCl_3$.	0.64 (1)
O	0.38 " $SrCl_2$.		127 (3)
P	0.38 " "	0.5 M NaCl.	94 (3)

* The efficiency coefficient was 108 in an experiment made with the same extract, or it was 119, an average of two determinations, in one of which a different extract was used.

efficients and to a similar degree. In both cases the addition of 2.0 M NaCl is more injurious than the addition of 0.5 M NaCl. Again this result can be explained, if we assume that addition of NaCl to the $CaCl_2$ extract changes the $CaCl_2$ combinations to the NaCl combinations, which latter possess a very low degree of activity and are irreversible. The same effect is obtained, if we add NaCl to $BaCl_2$ or $SrCl_2$ extracts (Table VIII, Experiments H, I, O, and P). Also subsequent addition of $MgCl_2$ to a $CaCl_2$

extract leads to a lowering of the coefficient and to an intermediate condition, similar to the results obtained in Tables VI and VII. Our data are insufficient at present, as far as a quantitative comparison, between the effects of addition of $MgCl_2$ and of NaCl to the $CaCl_2$ extract, is concerned. Table VIII shows, furthermore, that addition of salts of heavy metals to the $CaCl_2$ extracts is much more injurious than addition of NaCl, although it appears that the activity of urease is not entirely destroyed by the addition of salts of heavy metals.

SUMMARY AND CONCLUSIONS.

1. Whereas the salts of alkaline earths and in decreasing order $MgCl_2$ and $MnCl_2$ are efficient in the extraction of urease from amebocyte tissue of *Limulus*, salts with trivalent anions or cations are ineffective or very little effective.

2. While the salts of alkaline earth metals are, within a certain range of concentration, much more efficient as extractives than salts of alkali metals, in higher concentration they become very much more toxic than the latter; in higher concentration they stand in toxicity between the toxicity of salts of alkali metals and the salts of heavy metals. The salts of alkali metals on the other hand gain in efficiency, if used in higher concentration. It is probable that in this last case we do not have to deal with a specific chemical, but with an osmotic effect, inasmuch as solutions of non-electrolytes also become more effective in higher concentrations. The higher osmotic pressure perhaps prevents or retards certain injurious interactions between the enzyme and water.

3. There is an optimum on the acid as well as on the alkaline side, in the extraction of urease from amebocyte tissue by NaCl.

4. Whereas in accordance with our previous findings, we observed that addition of optimal amounts of $CaCl_2$ to NaCl extracts, or addition of a quantity of NaCl sufficient to produce an optimal concentration of NaCl, does not increase the activity of the extracts, addition of $CaCl_2$ to extracts made with $MgCl_2$ leads to an increase in the activity of the extract which is commensurate with the amount of $CaCl_2$ added.

5. If the mixtures of NaCl and $CaCl_2$ or of $MgCl_2$ and $CaCl_2$ are used for extraction, the results are somewhere intermediate be-

tween those of the two components of the mixtures; the efficiency coefficients are the higher, the greater the proportion of $CaCl_2$, and are the lower, the greater the proportion of $MgCl_2$ and especially of NaCl in the mixture. Addition of NaCl to $CaCl_2$ extracts produces a lowering of the coefficients of the extracts, which is the more marked the greater the quantity of NaCl added. Addition of salts of heavy metals almost destroys the activity of $CaCl_2$ extracts.

6. The lowering of the pH which we observe after addition of $CaCl_2$ to the extractive or to the extract probably depends largely on the formation of $CaCO_3$ as the result of the interaction of $CaCl_2$ and $(NH_4)_2CO_3$; there very likely takes place in addition an interaction between a buffer substance produced in the $CaCl_2$ extract and the alkali produced in the course of the urease action.

7. Tentatively we may assume that urease or a substance associated with the enzyme or a substance markedly influencing the activity of the enzyme combines with various cations and that the efficiency coefficients of the urease in these combinations differ greatly. While the Mg combination is not so potent as the combinations with Ca, Ba, or Sr, the former at least preserves the enzyme in such a condition that Ca can take the place of Mg in these combinations. Therefore, in a mixture of Ca and Mg salts, both Mg and Ca combinations apparently form side by side and the activity coefficients are thus intermediate. On the other hand, the combinations with the alkali metals seem to be of such a nature, that the enzyme is changed irreversibly and that subsequently an effective combination with Mg or Ca cannot be produced. It is therefore necessary to add the salt of the alkaline earth metal to the alkali metal salt before extraction, at a time when the alkali metal has not yet had a chance to enter into a combination with the enzyme or with associated substances, if we wish to raise the activity above that of the NaCl extract.

8. We may conclude from these data, that the specific effects of the salts in these experiments do not depend upon their action as extractives in the restricted sense, but upon their interaction with the enzyme or with a specific associated substance, after the latter has been liberated from the tissue.

9. It seems that we can distinguish at least three different modes of salt effects in the interaction of the salt with the urease. (1)

There are specific combinations of cations with the enzyme or with associated substances. In the case of combinations of this kind the alkaline earth metals are the most favorable cations. (2) There are injurious salt effects of a non-specific character. Salts of heavy metals are most injurious; salts of alkaline earth metals are less injurious than those of heavy metals, but more injurious than salts of alkali metals. (3) There are in all probability osmotic effects of salts. Increased osmotic pressure of salts or non-electrolytes, at least within the range which we have examined so far, acts favorably on the enzyme. There are, in addition to the salt actions, specific actions of proteins which may modify the effect of salts on the urease.

10. Our experiments suggest that at least to a certain extent, the salt actions which we observed are specific for the urease which is extracted from amebocyte tissue under special conditions of extraction, and that these specific relationships may neither apply generally to other enzymes extracted from amebocyte tissue, nor to urease extracted from other substances.

THE PLURAL NATURE OF VITAMIN B.

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Ever since the existence of vitamin B has been generally accepted, suggestions have appeared from time to time that this dietary factor is in reality a mixture, and not a single substance. The earlier literature on this topic has been summarized repeatedly, and citation of any but the more recent papers seems unnecessary at this time. The evidence has become continually more convincing, but the papers of Smith and Hendrick (1) and of Goldberger and collaborators (2) are the first that carried conviction to the greater body of readers. Smith and Hendrick examined two substances for vitamin B activity, a vitamin B picrate and autoclaved yeast. Neither alone was adequate as a source of vitamin B, but a combination of the two was fairly satisfactory. Goldberger and associates presented a greater body of evidence than Smith and Hendrick, but used essentially the same method. They observed that large quantities of either an alcohol extract of maize meal or a preparation of autoclaved yeast are inadequate as a source of vitamin B, but a combination of the two is effective.

Salmon (3) prepared similar factors from the leaves and seeds of the velvet bean and other plants, but by the use of fullers' earth obtained the active agents in a more concentrated form. In a later paper (4), these findings have been elaborated somewhat. A careful study of this topic has also been reported by Chick and Roscoe (5) who used methods similar to those just described. Their evidence also seems to indicate conclusively that vitamin B is a mixture. A number of other workers have made similar reports (6), in entire agreement with those just cited, but most of them are less elaborate and will not be described here in detail. It is probably unnecessary to point out that when a separation of the vitamin components has been attained, one is chiefly antineuritic, the other growth-promoting. Autoclaved preparations retain the latter factor, but are completely devoid of the former.

We believe then that vitamin B does contain at least two factors. It seems to us, however, that the evidence would be even more conclusive if a single preparation were used, in itself a complete source of vitamin B, but which with suitable manipula-

tion manifests on the one hand only antineuritic activity, on the other only growth-promoting activity. A mixture of these two modified preparations, however, should be a satisfactory source of vitamin B. The possibility of obtaining such preparations seemed realized when we observed (7) that a vitamin B preparation exposed to a quartz mercury arc retains its antineuritic activity, but that it loses some other essential property. Irradiated yeast or an irradiated vitamin B concentrate was effective in relieving pigeons from an acute attack of polyneuritis, but final collapse, due to the absence of some other essential, was the inevitable sequel. Autoclaved yeast, it will be recalled, loses its antineuritic potency, but as shown by other workers, retains growth-promoting activity. These facts suggest the possibility that vitamin B is a mixture of two components, and that one is destroyed by irradiation, the other by high temperatures. We decided, therefore, to study in more detail each of these modified preparations, and also to determine whether there is any supplementary relation between them.

As experimental animals, rats, chicks, and pigeons were used, and usually they were placed under observation at the earliest practicable age. In some series a few of the rats were only 21 days of age when the initial weights were taken, in others they were as much as 28 days old. In each series, however, the rats were, so far as possible, equally distributed between the various groups, according to age, sex, weight, and parentage. The chicks were newly hatched in every case, and the pigeons varied from 6 to 8 weeks in age by the time they had learned to consume readily our experimental diets. We were not able in every case to secure a sufficient number of pigeons of the same variety, but most of them were Silver Kings. A number of White Kings were also used, and a few Yellow Kings. In our experience the Silver Kings are hardiest and most suitable for this type of work.

With one exception the same ration, No. 465, was fed to all experimental animals. This diet is regarded as practically free from vitamin B, and has the following composition:

	per cent		per cent
Casein*	20	Cod liver oil	5
Corn-starch	58	Cellulose	3
Lard	10	Salts (8)	4

* Washed continuously for 1 week with dilute acetic acid.

One group of chicks, however, received its vitamin B supplement incorporated in the ration. The diet was identical with Ration 465, except that 15 parts of yeast had been added and an equal quantity of starch removed. This yeast component was, however, modified in various ways. Thus some chicks received untreated yeast, some a heated, some an irradiated preparation, while others received a mixture of equal parts of the two modifications.

Usually the food was kept before the animals continually to be consumed at will, but in one trial the pigeons were hand-fed to insure an equal intake of food by all groups. In order to facilitate the process of hand feeding, the various constituents of the ration, except the cod liver oil, were mixed, moistened, and after being moulded into small pellets, dried. These pellets were then coated evenly with the cod liver oil and kept in a cold room until used. The vitamin B supplements were always fed separately from the rest of the ration, except in the one instance just mentioned of the chicks. The daily portions of the supplement for the birds were placed in gelatin capsules and were hand-fed. The supplements for the rats were placed in glass dishes, and in practically every instance they were consumed immediately.

Two sources of vitamin B were used, Harris dried yeast, and a vitamin B concentrate prepared by ourselves from fresh brewers' yeast,[1] by the method of Osborne and Wakeman (9). Our experience with commercial concentrates has not been satisfactory, and we regard negative results obtained with them as of doubtful significance.

Prior to irradiation the material was ground to a fine powder and scattered in a thin layer, not over 0.5 mm. thick, on a glass plate 25 × 30 cm. The plate was directly below the quartz mercury arc,[2] at a distance of 25 cm., and each preparation was exposed for a period of 10 hours. We are unable to state quantitatively the amount of radiant energy per unit of area of the exposed material, but the lamp gives a brilliant spectrum ex-

[1] We take this opportunity to express our obligation to Dr. O. F. Steidemann, Chief Chemist of the Anheuser Busch Company, of St. Louis, who kindly placed at our disposal several hundred pounds of brewers' yeast.

[2] The arc is manufactured by the Cooper Hewitt Electric Company, and operates at 117 volts and 3.8 amperes.

tending far into the ultra-violet region. The temperature of the irradiated materials varies somewhat, depending on the temperature of the room, but the highest reading observed was 40.1°.

The preparations to be heated were thoroughly moistened with water, heated for 2 hours in an autoclave at 120°, then dried and powdered.

In practically every series of observations, the animals were divided into four groups, corresponding to the four modifications of the vitamin that were used. The first group received the autoclaved supplement, and the second received the irradiated material. Each member of Group 3 received one-half as much of the autoclaved supplement as did the individuals of Group 1, and in addition one-half as much of the irradiated supplement as did the individuals of Group 2. Group 4 received the untreated preparation. The total amount of supplement supplied to the individuals of the various groups was the same.

The rats were confined in small individual cages, made of hardware cloth, with raised screen bottoms. The pigeons and chicks were confined by the group method. Since the chicks were placed on the experimental rations immediately after hatching, they were kept in electrically heated brooders until 4 weeks of age, to assure a suitable temperature during that critical period.

In our first trials yeast itself was used, and though the results were decisive, we believe the vitamin concentrate is much more suitable for experimental purposes. Some of our observations with yeast are presented in Fig. 1.

It will be noted that the mortality on both of the modifications, either heated or irradiated yeast, was 100 per cent. The survival period on the irradiated yeast was a little longer than on the autoclaved material, but we attach no significance to that, other than indicating that the reaction due to exposure may not have gone to completion.

Our observations on chicks were less satisfactory, due chiefly we believe, to the fact that their nutritional requirements (10) are partially unknown as yet. Occasionally a group will grow at an almost normal rate on a synthetic ration, but that is rare, and the variability is always great. We have, therefore, selected a group in which the controls made satisfactory growth, and have incorporated the data in Fig. 2.

It will be noted that the chicks receiving the mixture of irradiated and autoclaved yeast did as well as those receiving untreated yeast, and much better than those receiving either the irradiated or autoclaved material alone.

From our standpoint the behavior of the chicks that received autoclaved yeast was very interesting. Two of them did as well as chicks usually do on untreated yeast, and they were in good condition when removed from observation at 9 weeks.

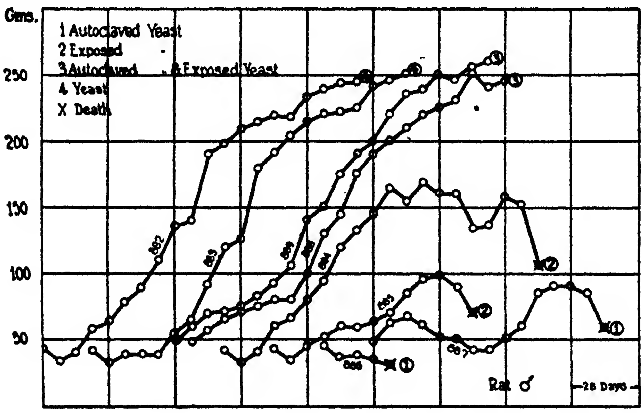

Fig. 1. This chart shows that for rats neither autoclaved yeast nor irradiated yeast (Groups 1 and 2) is an adequate source of vitamin B. The mixture (Group 3) of these preparations is, however, not much less effective than the untreated material (Group 4). The supplements were fed at the following levels daily: from the 7th day, 0.2 gm.; from the 21st day, 0.4 gm.; from the 28th day, 0.5 gm.; from the 42nd day, 0.6 gm.; from the 56th day, 0.75 gm.; from the 70th day, 1 gm.; from the 105th day, 1.25 gm.

In the next series rats were employed, and the yeast concentrate was given, as shown in Fig. 3. The response was much more prompt than when yeast was used. The rats receiving the autoclaved concentrate lost weight almost from the beginning and succumbed within 5 weeks at the latest. The loss of weight of those receiving the irradiated fraction was less marked, but the final outcome was the same. In contrast with these, however, the rats receiving the mixture of autoclaved and irradiated fractions did practically as well as those that received the untreated fraction.

Lastly we are presenting in Figs. 4 and 5 the results obtained with some of the pigeons that received the vitamin concentrates. Those in Fig. 4 were fed *ad libitum,* and those in Fig. 5 all received the same amount of food per unit of weight.

It will be observed in both instances that all pigeons receiving either the irradiated or autoclaved concentrate lost weight and succumbed shortly, none surviving longer than 7 weeks. Those

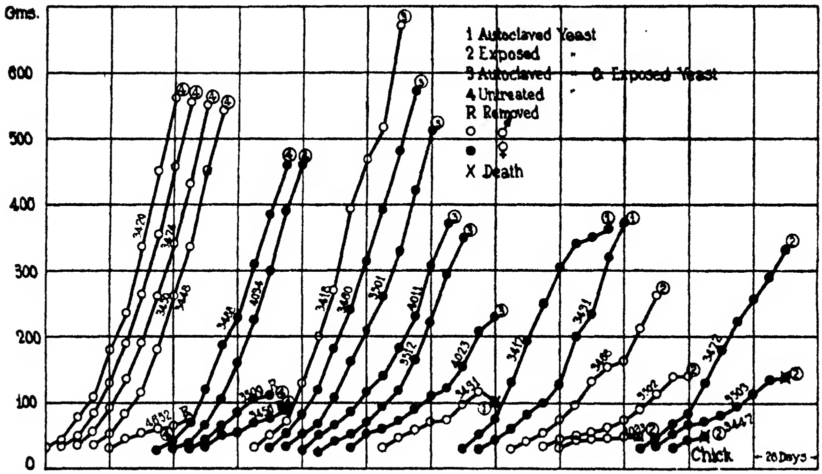

Fig. 2. The chicks receiving the mixture (Group 3) of treated yeast supplements did as well as those that received the untreated material (Group 4). Either of these preparations is apparently more suitable than is either the autoclaved (Group 1) or the irradiated material (Group 2). In this series the vitamin B supplements constituted 15 per cent of the ration. Originally there were seven chicks in each of Groups 1, 2, and 3. We have omitted, however, all those that died within the first 2 weeks.

receiving the mixture of the autoclaved and irradiated fractions, however, did as well as those receiving the untreated supplement.

It will be noted that the groups receiving either the untreated material or the mixture, shown in Fig. 4, either maintained their weight or made some increase. Similar groups in Fig. 5 were on a restricted food intake, and these at first suffered large losses in weight and then seemed to come to equilibrium. The continued rapid loss of weight in the groups receiving singly the autoclaved or irradiated preparations (Fig. 5) was unexpected, however, for

owing to hand feeding they were receiving more food at the last than similar birds shown in Fig. 4 would consume voluntarily. Inspection of the cages yielded only slight evidence of regurgitation, though we cannot be positive that it did not occur. In similar observations Marrian, Baker, Drummond, and Woollard (11) estimate that not over 20 per cent of the forcibly fed food was regurgitated. Their birds, however, suffered little if any loss in weight. These observers also reported spontaneous cures from polyneuritis, but we did not note such cases. Temporary relief

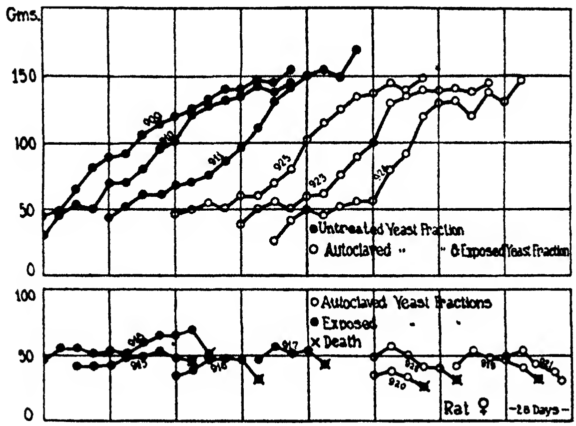

FIG. 3. All animals in this series received the vitamin B concentrate. The mixture of irradiated and autoclaved fractions was little if any less effective than the untreated material. When fed alone neither the autoclaved supplement not the irradiated supplement was adequate as a source of vitamin B. For the first 21 days, all animals received 50 mg. of the concentrate, and then the amount was increased to 80 mg. This was increased to 150 mg. on the 42nd day, and to 200 mg. on the 70th day.

from acute attacks was frequently observed, but there were no real recoveries. Possibly this difference in behavior may be explained by the immaturity of the pigeons we were using.

Since we believe the deaths of the pigeons receiving autoclaved and irradiated vitamin B supplements were due to different inadequacies, the symptoms preceding collapse are of some interest. Until shortly before death there were no perceptible differences in their behavior. They became apathetic, and were in an obviously poor nutritional condition, but at the end the

majority of those receiving the autoclaved preparations developed symptoms of polyneuritis, with a typical head retraction. Many, however, did not display characteristic symptoms of any kind. Of those receiving the irradiated fraction, a few gave some indication of head retraction, but practically all of them passed into a stupor which soon ended in death.

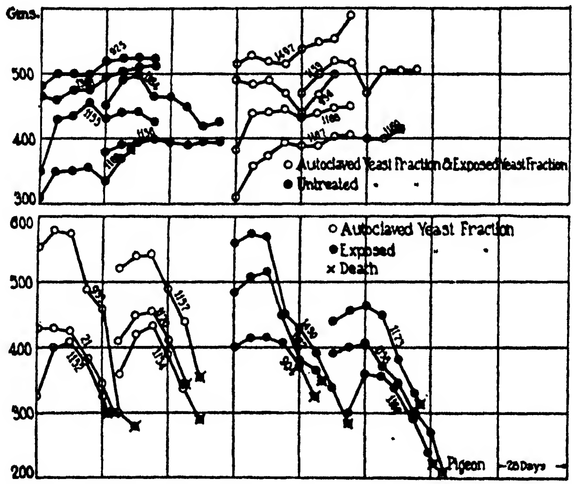

FIG. 4. This chart shows the effect of high temperature and of exposure to ultra-violet rays, on the concentrate when used as a source of vitamin B for pigeons. The mixture of these concentrates was apparently as active as the untreated material. When fed alone neither the autoclaved supplement nor the irradiated supplement was adequate as a source of vitamin B. For the first 8 days all the pigeons received an adequate ration of natural foodstuffs, but powdered finely so as to resemble our synthetic rations. On the 9th day they were given Ration 465, and 200 mg. daily of the supplements as described above. On the 23rd day the amount of supplement was increased to 300 mg. daily. The concentrate was fed separately, by hand, in all cases.

In order to show more clearly the supplementary relation between the irradiated and autoclaved vitamin B carriers, we are presenting in Table I a brief summary of our data. We are omitting, however, the series of chicks (Fig. 2) which received yeast incorporated in the ration as a source of this supplement, since our observations were discontinued before any evidence was obtained as to the length of the survival period.

Table I shows that when either irradiated or autoclaved materials are offered as the sole source of vitamin B, there are no survivors. However, the mixture of supplements was adequate, although the percentage of survivors was not always 100. Since, however, the different species do not behave alike in feeding trials, we shall consider them separately. Rats are much the

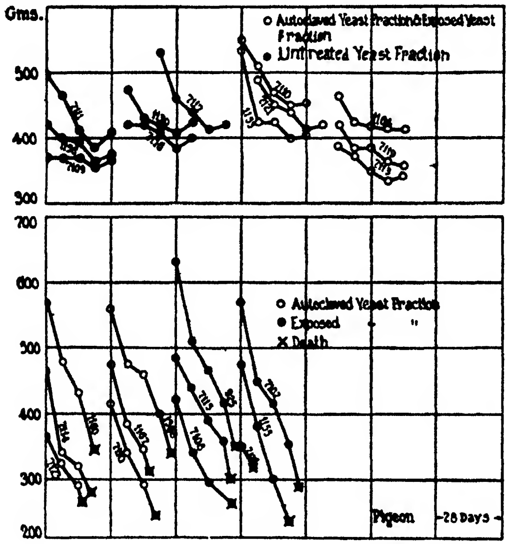

FIG. 5. The results shown in this chart are in entire agreement with those given in Fig. 4. The squabs shown in Fig. 4 were fed *ad libitum*, while those described in Fig. 5 were hand-fed, all receiving the same amount of food per unit of weight. For the first 14 days each individual received daily 1 gm. of food for each 20 gm. of body weight. On the 15th day this quantity was changed to 1 gm. of food for each 30 gm. of body weight. The amount of supplement given was unchanged, 200 mg. daily for each individual.

simpler to handle, chiefly because their nutritional requirements are better understood and because they consume readily almost any adequate and suitable diet. Squabs require some training before they will eat at all, and our experience is that they require a second training before they will eat the finely powdered synthetic rations we employ. We did not appreciate this fact until the first experiment was under way, and we believe most of this group

would have starved on rations of this type, even if the diets were entirely adequate. In later trials the birds were all given an adequate ration, similar in appearance to our experimental diets, until their weights made it clear they had learned to eat. Many were hand-fed until they were satisfactorily trained, to prevent voluntary starvation. Feeding trials of this nature with chicks are uncertain at present, owing to the limitations of our knowledge of their nutritional requirements, and the variability within groups is always perplexing.

TABLE I.

Effect of Heat and of Exposure to Ultra-Violet Rays, on Vitamin B Component of Diet.

Supplement used.*	Experimental animal.	No. of animals.	Percentage of survivors.
Irradiated.	Rat.	15	0
	Pigeon.	27	0
	Chick.	13	0
Autoclaved.	Rat.	15	0
	Pigeon.	27	0
	Chick.	13	0
Irradiated + autoclaved.	Rat.	14	100
	Pigeon.	27	70
	Chick.	12	42
Untreated.	Rat.	9	89
	Pigeon.	21	76
	Chick.	13	61

* This includes both yeast and the Osborne-Wakeman concentrate.

To return then to the feeding trials with the mixture of supplements, it will be noted that it is at least a fairly adequate source of vitamin B. Of the rats on this diet, all were making some growth, and were in a satisfactory nutritional state when all their companions receiving the supplements separately had succumbed. In contrast to the rats, there were several mortalities among the pigeons and chicks receiving the mixture of vitamin concentrates. The percentages of survivors among the control groups were not much larger, however, and we do not regard the differences as necessarily significant.

A few observations were made on the body temperature of pigeons receiving, in some cases autoclaved, in others irradiated supplements. These records are scattered and limited in number, but they indicate distinct differences in physiological behavior. The body temperature of those receiving the autoclaved supplement was markedly lowered at least a week before the onset of characteristic symptoms. In one case a pigeon seemed to be in a low nutritional state and was found to have a body temperature of 102.8°. 7 days later, a few hours before the bird died, a reading of 100.3° was obtained. Another pigeon gave a reading of 104.0° 8 days before death, and 102.4° on the last day it was observed alive. In a third case the temperature on the day preceding death was 99.0°. Similar reports on the effect of a deficiency of the antineuritic vitamin have been made by others (11, 12).

Apparently the effect of the irradiated supplement on the heat-regulating mechanism is quite different, as the lowest reading observed, taken the day preceding death, was 104.4°. Two other readings were taken the last few days before death, one of 106.5°, the other of 107.6°. The body temperature of pigeons receiving the mixture of supplements ranged from 105.0–108.5°.

In view of the confusion that now exists in the terminology of vitamin B, a few comments may be pertinent. In the first place, we regard the designation "growth-promoting," so frequently applied to one of the vitamin B constituents, as unsuitable. Indirectly it does promote growth, by making possible a more normal nutritional condition, but in this sense, the antineuritic, the antiophthalmic, the antiscorbutic, and the antirachitic vitamins are also growth-promoting. Until we have some understanding of the chemical structure of the vitamins, or until we have more definite knowledge of their functions, a logical terminology is impossible. We believe, however, that at present the term "antidystrophic" is less objectionable, and less misleading, than is growth-promoting. From our point of view it seems desirable, for the present, to retain the nomenclature now generally in use, and to amplify it as new facts become available. We suggest, therefore, that in the future the term vitamin B should include only the antineuritic factor; and that the so called growth-promoting substance be designated as vitamin F.

SUMMARY.

1. Under the experimental conditions we observed, vitamin B carriers become inadequate after exposure to ultra-violet rays for a period of 10 hours.

2. These irradiated materials retain antineuritic activity, but lose their so called growth-promoting properties.

3. Irradiated and autoclaved vitamin B preparations are both inadequate, when used as the sole source of the vitamin. There is a supplementary relation between them, however, and a mixture of the two is a potent source of vitamin B.

4. Vitamin B, as the term has been used in the past, is a mixture of at least two distinct vitamins.

5. It is suggested that the designation vitamin B be reserved for the antineuritic factor; the so called growth-promoting factor should be given an individual designation and be known as vitamin F.

BIBLIOGRAPHY.

1. Smith, M. I., and Hendrick, E. G., *Pub. Health Rep., U. S. P. H. S.*, 1926, xli, 201.
2. Goldberger, J., Wheeler, G. A., Lillie, R. D., and Rogers, L. M., *Pub. Health Rep., U. S. P.H. S.*, 1926, xli, 297. Goldberger, J., and Lillie, R. D., *Pub. Health Rep., U. S. P. H. S.*, 1926, xli, 1025.
3. Salmon, W. D., *J. Biol. Chem.*, 1927, lxxiii, 483.
4. Salmon, W. D., Guerrant, N. B., and Hays, I. M., *J. Biol. Chem.*, 1928, lxxvi, 487.
5. Chick, H., and Roscoe, M. H., *Biochem. J.*, 1927, xxi, 698.
6. Hassan, A., and Drummond, J. C., *Biochem. J.*, 1927, xxi, 653. Sherman, H. C., and Axtmayer, J. H., *J. Biol. Chem.*, 1927, lxxv, 207. Williams, R. R., and Waterman, R. E., *Proc. Soc. Exp. Biol. and Mea.*, 1927, xxv, 63. Eddy, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 125. Daniels, A. L., and Brooks, L., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 161. Rosedale, J. L., *Biochem. J.*, 1927, xxi, 1266.
7. Hogan, A. G., and Hunter, J. E., *J. Biol. Chem.*, 1928, lxxviii, p. xvii.
8. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.
9. Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1919, xl, 383.
10. Hogan, A. G., Guerrant, N. B., and Kempster, H. L., *J. Biol. Chem.*, 1925, lxiv, 113.
11. Marrian, G. F., Baker, L. C., Drummond, J. C., and Woollard, H., *Biochem. J.*, 1927, xxi, 1336.
12. Dutcher, R. A., *J. Biol. Chem.*, 1918, xxxvi, 63.

STUDIES IN MUSCULAR ACTIVITY.

V. CHANGES AND ADAPTATIONS IN RUNNING.

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This paper reports experiments on a normal man for a period of about 1 month. During this time the diet was maintained constant except for occasional variations in the ingestion of glucose and phosphate. Other conditions of life were also maintained nearly constant except that the subject frequently ran on a treadmill at various speeds and for various lengths of time. Throughout the experiments quantitative observations were made to determine the main variations of the metabolism, and during each bout of work the metabolism and the properties of the blood, as well as their variations as functions of time and of metabolic rate, were studied in detail.

In the course of investigations carried on in the laboratories of the Massachusetts General Hospital, we have previously established certain facts concerning: (1) the changes in the circulatory and respiratory mechanisms during work; (2) the responses of different normal individuals to work of a given character; (3) the operation of the body in a steady state. The investigation now to be described amounts to an extension by experiment of our previous observations.

For the steady state the highest metabolic rate obtainable on the bicycle ergometer during previous experiments was 2.5 liters per minute of oxygen consumption for less than 30 minutes. We now find that an untrained subject, J. H. T., who had never maintained on the bicycle a steady state when the consumption of oxygen was more than 2.3 liters per minute, may attain to an

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oxygen consumption of over 3.0 liters per minute for 17 minutes, and of 2.7 liters per minute for more than an hour and a half, meanwhile remaining in a steady state.

The experiment was conducted as follows: In the beginning a period of 5 days was devoted to the establishment of nitrogen equilibrium, the existence of which was proved by determinations of nitrogen in the urine. This condition depended upon the ingestion of a constant diet, consisting of 124 gm. of protein, 203 gm. of carbohydrate, 154 gm. of fat, 1600 gm. of water, and 3 gm. of sodium chloride. The diet was made up daily from the same quantities of the same foodstuffs. It is equivalent, according to Sherman's tables (1), to 2780 calories. After this preliminary period, urinary constitutents were determined daily (Table VIII).

All work, except that incidental to sedentary life, was done on a motor-driven treadmill[1] operated in a horizontal plane. In successive experiments the speed of the treadmill was varied, and with it the rate of work done by the subject. With this treadmill the subject did not perform any work in driving the belt; all his energy was expended in keeping pace with the tread and in the necessary attendant movements.

With the exception of Experiment 1, the bouts of work were begun 2½ hours after breakfast. Venous blood was drawn from the arm before and near the end of work. In some cases blood was also taken during the bout and several minutes after work stopped.

To the blood thus obtained heparin was added as an anticoagulant and analyses were made to provide data for the construction of carbon dioxide dissociation curves and to determine the various organic and inorganic constituents of the blood.

The volume of cells expressed as the per cent of the volume of whole blood was obtained from equilibrated blood which had been centrifuged for 1 hour at 3000 R.P.M. Serum water and cell water were determined gravimetrically. Lactic acid was determined for whole blood by the procedure of Friedemann (3). The method of Fiske and Subbarow (4) was used for inorganic

[1] We are indebted to Dr. F. G. Benedict for the loan of this treadmill which is the one long in use in the Nutrition Laboratory of the Carnegie Institution of Washington, Boston. It has been fully described by H. M. Smith (2). We express our hearty thanks to Dr. Benedict for this generous assistance.

phosphorus in the serum. Calcium was determined by Clark and Collip's modification of the Kramer and Tisdall method (5). The concentration of base in the serum and cells was determined by the method of Fiske (6). Urea nitrogen was determined with the aid of the manometric gas apparatus as described by Van Slyke (7). For the determination of serum protein, 2 cc. of serum were subjected to the macro-Kjeldahl procedure. From the total nitrogen the non-protein nitrogen was subtracted. The remainder was multiplied by the usual factor 6.25.

The following methods of analysis were employed for the determination of the urinary constituents: hydrogen ion concentration, Clark (8); chloride, Folin (9); sulfate, Fiske (10); total acid, Folin (9); fixed base, Fiske (6); ammonia nitrogen, Folin (9); creatinine and creatine, Folin (9); urea nitrogen was determined by Van Slyke's method (11). Total nitrogen was determined by a macro-Kjeldahl method. Inorganic phosphates were determined by a uranium acetate method to be described later by one of us (A. F.).

During a bout outdoor air was inhaled through a valve and the expired air was collected from time to time in a 600 liter Tissot gasometer. The valves, the method of collecting expired air, and the method for determination of blood flow have been described in an earlier paper (12). Body weight was measured daily as well as before and after each bout of work.

Three rates of work were studied, walking at 3.2 miles per hour, running at 6.4 miles per hour, and running at 9.8 miles per hour. They involved oxygen consumption of about 0.95, 2.3, 3.0 liters per minute.

Tables I to VI show that a steady state is soon reached after the beginning of work. The last bout (Table VI) was too severe to be prolonged, and the oxygen consumption did not reach a steady state. In this bout the subject was compelled to stop because of exhaustion.

Phosphate was taken before three bouts of the second type. In all instances 10 gm. of crystallized monosodium phosphate were ingested in the 12 hour period previous to exercise. In addition to the phosphate 250 gm. of dextrose were taken ½ hour before Experiment 3. In comparison with the bouts in which no phos-

TABLE I.

Experiment 1.

Walking for 6 hours, 36 minutes, at a speed of 3.2 miles per hour.

Time elapsed.	Pulse rate.	Respiratory rate.	Ventilation.	O_2 used.	R. Q.	O_2 used per heart beat.	Alveolar pCO_2.	Oxygenated venous pCO_2.	Blood flow.	Venous saturation.	Stroke volume.
min.			*l. per min.*	*cc. per min.*		*cc.*	*mm. Hg*	*mm. Hg*	*l. per min.*	*per cent*	*cc.*
0	Work began.										
2	120		22	1222	0.75	10.2					
5		24	25	890	0.86	7.7					
10	112		25	892	0.83	8.0	33.7	56.0	8.6		80
32	104	24	21	902	0.80	7.8	36.3	55.0	9.2	59.2	
37	114	23	22	864	0.82	7.6	34.0				
52	110		24	990	0.78	9.0	33.8	55.0	10.0	58.1	91
56	104		25	971	0.79	8.8	33.6	55.3	9.8		94
69	102		25	908	0.77	9.1					
81	100	25	24	926	0.80	9.3	33.6			57.7	
105	96	24	25	907	0.80	9.5	36.5				
115	96		24	894	0.81	9.3				57.7	
137		23	25	923	0.81	9.8	34.4	53.5	9.5		99
150	96	27	26	974	0.76	10.2				57.2	
179		29	24	912	0.80	9.5				56.8	
189	96	28	23	924	0.80		34.4			56.3	
206	97		26	937	0.79	9.7					
218		31	25	919	0.81	9.6	34.4	53.8	10.4		107
249			23	897	0.78	9.5				55.4	
261	94	26	25	922	0.76	9.8	32.8	53.8	11.2		119
317	101		25	959	0.79	10.2	30.6	50.6	10.5	54.9	118
329	92	26	25	974	0.77	10.3	33.5	51.2	11.1		110
332							32.5	51.2	11.4	54.9	116
370	96		27	960	0.77	9.6	33.5	50.0	12.3	54.0	130
384							33.3	50.9	11.4		118
396	Work stopped, blood drawn.										
397	104		19	568	0.83						
398½	94		16	528	0.80						
404	94		13	362	0.80						
408	90		11	288	0.74						
414	87		11	296	0.78						
427			11	284	0.81						
436	80		12	334	0.73						
455	80		9	246	0.72						

phate was taken, nothing was noted subjectively or objectively which could be attributed to the salt. Throughout Experiment 3 the subject experienced marked nausea.

TABLE II.

Experiment 2.

Running for 2 hours, 6 minutes, at a speed of 6.4 miles per hour; 10 gm. NaH_2PO_4 during the 12 hours preceding the exercise.

Time elapsed.	Pulse rate.	Respiratory rate.	Ventilation.	O_2 used.	R.Q.	O_2 used per heart beat.	Alveolar pCO_2.	Oxygenated venous pCO_2.	Blood flow.	Venous saturation.	Stroke volume.
min.			*l. per min.*	*cc. per min.*		*cc.*	*mm. Hg*	*mm. Hg*	*l. per min.*	*per cent*	*cc.*
0	Work began.										
1½	140		46	2230	0.89	16.0					
6	140	34	53	2285	0.92	16.3					
9	140	42	54	2271	0.96	16.2	37.7	72.0	18.0	32.3	117
19	158	41	54	2164	0.88	15.5	37.4	72.9	15.7		100
29	166	40	56	2364	0.85	14.2	36.9	68.2	18.4	32.7	111
39	174	41	57	2270	0.86	13.0	35.8	67.2	18.3	32.7	105
51	172	44	57	2280	0.87	13.2	35.4	67.5	18.3		106
59	176	44	56	2258	0.83	12.8	34.5	66.1	17.7	34.1	100
68	168		54	2220	0.83	13.3				33.1	
78	172		56	2455	0.79	14.3					
88	176	42	58	2440	0.80	13.9	36.1	67.2	18.6	32.7	105
99	176	40	54	2344	0.79	13.3		67.4	18.0	31.8	100
116	174		55	2285	0.84	13.3	35.7	64.4	19.1	31.8	111
124	Work stopped, blood drawn.										
124½	160		46	1404	0.81						
125½	150		37	934	0.90						
128½	146		24	383	1.01						
139	110		10	422	0.69						
154	100		8	334	0.62						
172			8	329	0.67						
185			8	302	0.68						

In all three grades of work the respiratory quotient goes through a characteristic rise in the first few minutes of work, then falls to a somewhat lower value. In Experiments 1 and 2 this was followed by a gradual lowering of the respiratory quotient throughout the period of work. In Experiments 3, 4, and 5 (Fig. 1) the values

remained constant during the period of about 80 minutes following the original fluctuation and then during the last half hour of work gradually declined; the respiratory quotients of Experiment 6 are not comparable with those of the other experiments since a steady

TABLE III.

Experiment 3.

Running for 2 hours at a speed of 6.4 miles per hour; 10 gm. of NaH_2PO_4 during the 12 hours preceding the exercise; 250 gm. of dextrose 30 minutes preceding the exercise.

Time elapsed.	Pulse rate.	Respiratory rate.	Ventilation.	O_2 used.	R. Q.	O_2 used per heart beat.	Alveolar pCO_2.	Oxygenated venous pCO_2.	Blood flow.	Venous saturation.	Stroke volume.
min.			*l. per min.*	*cc. per min.*		*cc.*	*mm. Hg*	*mm. Hg*	*l. per min.*	*per cent*	*cc.*
0	Work began.										
1½			46	2005	0.79						
4½	170	38	54	2152	0.96	12.4					
9	174	38	57	2162	0.95	12.4	39.7	66.8	19.7	42.8	116
26	172	40	62	2400	0.88	14.0	39.0	65.0	20.5	40.0	119
40	162	44	61	2360	0.87	14.5	37.4	68.5	17.0	39.6	106
51	160	36	56	2318	0.90	14.4	40.5	68.3	19.1	39.6	119
65	160	44	63	2490	0.88	15.5	38.4	68.5	19.1	38.7	119
79	164	40	59	2390	0.88	14.5	40.5	68.5	19.0	37.8	118
97	170	37	59	2402	0.87	14.1	38.9	69.8	17.1	37.8	100
111	164	38	59	2410	0.89	14.1	39.6	69.4	17.9	36.4	100
120	Work stopped, blood drawn.										
120½	154		50	1350	0.98						
122½	138		24	600	0.93						
125	122		15	476	0.76						
133	106		8	335	0.74						
145	100		8	329	0.73						
164	94		8	329	0.76						
190	95		9	330	0.85						
221			9	312	0.85						

state was not reached at any time. In the recovery period there is similarly an initial sharp rise to a peak even higher than in the beginning of work. This is followed by a rapid fall to a minimum below the value for the resting condition. In the present experiments the occurrence of the two peaks in the graph of the respira-

TABLE IV.

Experiment 4.

Running for 2 hours, 3 minutes, at a speed of 6.4 miles per hour.

Time elapsed.	Pulse rate.	Respiratory rate.	Ventilation.	O_2 used.	R. Q.	O_2 used per heart beat.	Alveolar pCO_2.	Oxygenated venous pCO_2.	Blood flow.	Venous saturation.	Stroke volume.
min.			*l. per min.*	*cc. per min.*		*cc.*	*mm. Hg*	*mm. Hg*	*l. per min.*	*per cent*	*cc.*
0	Work began.										
1½	140	18	50	2100	0.88	15.0					
4	144		55	2115	0.93	15.1					
9	156	38	54	2090	0.93	14.9	38.1	70.2	16.7	34.0	102
22	152	41	57	2240	0.88	13.4	39.1	69.8	17.6	34.0	118
32			56	2220	0.89	14.0					
36	154	44	56	2200	0.88	14.3	37.9	67.4	18.1	34.4	118
47			57	2240	0.88	14.3					
51	162	46	57	2240	0.88	14.3	38.2	69.5	17.2	35.8	106
60			57	2290	0.90	14.0					
64	164	44	57	2280	0.89	13.9	38.0	69.8	18.5	36.7	115
72			56	2170	0.90	13.1					
76			56	2270	0.90	13.7	39.4	71.4	16.1	37.6	100
85		46	58	2290	0.91	13.8					
89	162		58	2280	0.88	13.7	37.7	67.9	20.2	37.2	124
98			55	2371	0.87	14.6					
102			55	2318	0.87	14.3	39.1	66.0	19.2	37.2	119
113			59	2425	0.84	14.6					
116	160	46	59	2365	0.88	14.5	37.8				
123	Work stopped, blood drawn.										
123½	148		42	1242	0.90						
125	142		25	688	0.70						
127½	130		19	516	0.75						
138	94	18	8	326	0.73						
156			8	293	0.70						
166			8	260	0.79						
185	91	16	8	260	0.79						
199			8	287	0.71						

tory quotient is independent of the grade of work, while the height of the rise is a function of the severity of work. It should be noted that during rest a small difference in absolute magnitude of carbon dioxide and oxygen turnover produces a relatively large

change in the value of the respiratory quotient, because the metabolic rate is low.

TABLE V.

Experiment 5.

Running for 2 hours, 1 minute, at a speed of 6.4 miles per hour; 10 gm. NaH_2PO_4 during the 12 hours preceding the exercise.

Time elapsed.	Pulse rate.	Respiratory rate.	Ventilation.	O_2 used.	R. Q.	O_2 used per heart beat.	Alveolar pCO_2.	Oxygenated venous pCO_2.	Blood flow.	Venous saturation.	Stroke volume.
min.			*l. per min.*	*cc. per min.*		*cc.*	*mm. Hg*	*mm. Hg*	*l. per min.*	*per cent*	*cc.*
0	Work began.										
1			40	1836	0.72						
3	158		50	2140	0.89	13.5					
6	158		54	2302	0.89	14.6					
8			53	2230	0.89	14.1					
13	168	43	58	2320	0.92	14.7					
18	168		57	2254	0.90	13.4	38.9	67.5	17.5	34.4	104
28	166	44	57	2275	0.90	13.5	38.5	65.0	19.2	34.4	115
38	164		56	2239	0.90	13.6					
48	160	46	58	2300	0.90	14.4	38.1	66.7	17.9	34.4	112
57	160	43	58	2332	0.90	14.6	38.0	65.7	18.6	33.8	116
65	170	42	57	2310	0.90	13.6	38.0	65.2	18.7		110
75	164	45	56	2242	0.90	13.7				33.8	
86	166	43	57	2400	0.88	14.4	38.2	66.7	18.3	32.8	110
96	168	47	60	2382	0.88	14.2	37.2	64.4	19.0	29.6	113
105	158		61	2421	0.85	15.2					
112			61	2480	0.82	15.7	37.4	65.9	17.6	25.0	111
118	158		58	2441	0.81	15.5					
121	Work stopped, blood drawn.										
121½	156		52.6	1536	0.96						
123	152		38	760	1.03						
125	142		33	856	0.78						
128	138		23	640	0.70						
133	100		11	470	0.64						
141	94		8	324	0.68						
154	92		8	296	0.72						
177	86		8	285	0.73						
192	82		8	288	0.81						

Taking these facts into account, one may divide both the period of work and that of recovery into two lesser periods. In

the beginning of work there takes place an adjustment of the body to the changed conditions. This is approximately complete in less than 15 minutes and is followed by a steady state. In the preliminary period of adjustment rapid increases occur in the oxygen capacity and lactic acid content of the blood, and there is a concomitant fall in the carbon dioxide pressure of arterial blood.

TABLE VI.

Experiment 6.

Running for 13 minutes at a speed of 9.8 miles per hour.

Time elapsed.	Respiratory rate.	Ventilation.	O_2 used.	R. Q.
min.		*l. per min.*	*cc. per min.*	
0	Work began.			
½	38	46	1775	0.80
1½		70	2540	1.08
3	42	77	2970	1.03
4	46	80	2980	1.07
6		83	2890	1.08
9	52	84	3120	0.97
13	Work stopped, blood drawn.			
13½		78	2310	1.06
14		64	1300	1.30
15		52	878	1.26
16		36	569	1.22
17½		32	524	1.14
19		31	434	1.25
21½		27	475	1.05
23		24	382	1.01
30		12	307	0.87
38		10	324	0.73
58		8	300	0.72
78		8	297	0.71
94		9	291	0.78

During moderate work (Experiments 2 to 6) there is little further change in the composition of the blood. The sharp rise in the respiratory quotient at the beginning of recovery is probably related to two factors: the call for oxygen ceases abruptly because there is little oxygen debt, while the adjustment of the excretion of carbon dioxide is slower and this substance is excreted in

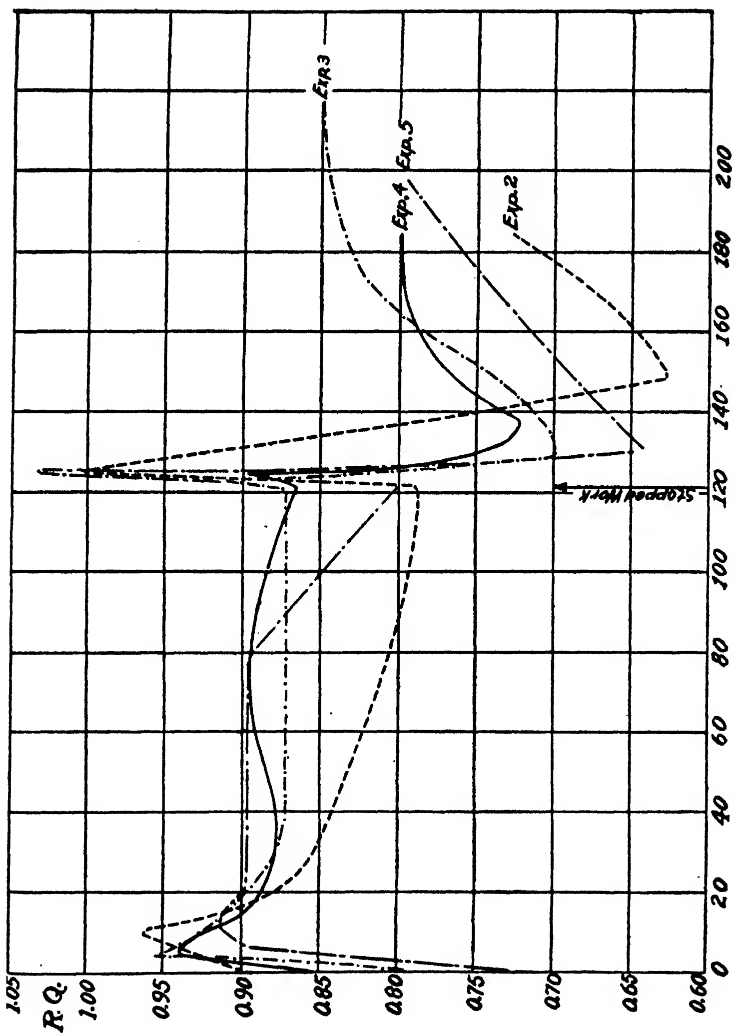

Fig. 1. Respiratory quotients at an oxygen consumption of 2.3 liters per minute.

relatively larger amounts during the period of readjustment. This continued high excretion of carbon dioxide is perhaps associated with the continued diffusion of lactic acid from the muscles into the blood stream and its passage into other parts of the body in which its oxidative removal is prolonged for some time. The second phase of recovery is marked by a very low respiratory quotient with a gradual rise to the value characteristic of the state of rest. No doubt this is in part related to the restoration of the resting level of blood bicarbonate. In all cases at least an hour was required for the respiratory quotient to return to the value 0.80. During recovery, at rest, the ventilation of the lungs returned to normal somewhat sooner than the oxygen consumption, which reached the resting level only after 2 hours and only in the walking experiment. In the other bouts the consumption of oxygen was 8 to 15 per cent greater after an hour of recovery than in the basal state.

The fall of the respiratory quotient during the later stages of work must depend upon decreased utilization of carbohydrate and increased utilization of other substances in total metabolism. Possibly the fact that this change is associated with increased consumption of oxygen and constant elimination of carbon dioxide may be due to slightly greater efficiency in the utilization of carbohydrate.

During the period of recovery, the long continued utilization of oxygen in amounts significantly larger than those characteristic of the so called basal condition calls for comment. We suggest that it should be regarded as the result of two processes: (1) the completion of the oxidation of substances already in process of utilization at the moment when work ceased (oxygen debt in the strict sense of the term); (2) the gradual restoration of the original condition of the body—so far as possible in the absence of food—by means of partial and incomplete oxidation of existing components. Manifestly the carbohydrate stores have been depleted; therefore it seems not improbable that a formation of carbohydrate from fat is the principal factor in this process.

Dextrose was ingested before Experiment 3 with the desire to note any change in the respiratory quotient that might follow. The only change observed was the failure of the respiratory

TABLE VII.

Observations on Blood Constituents before and after Exercise.

Experiment No.	Remarks.	O_2 used.	O_2 capacity.	$\frac{V_c}{V_b}$	$(H_2O)_s$	$(H_2O)_c$	$(BHCO_3)_s$, $pCO_2 = 40$ mm.	Arterial pH_s.	$(BHCO_3)_b$, $pCO_2 = 40$ mm.	Δ $(BHCO_3)_b$, from rest to work, $pCO_2 = 40$ mm.	$(Lactate)_b$.	Δ $(lactate)_b$, from rest to work.	Inorganic P.	Ca	Total fixed base.	Total fixed base.	Urea N.	Non-protein N.	Total N.	Protein.
		cc. per min.	*vol. per cent*		*gm. per l.*	*gm. per l.*	*vol. per cent*		*vol. per cent*	*m.-eq. per l.*	*m.-eq. per l.*	*m.-eq. per l.*	*mg. per 100 cc. serum*	*mg. per 100 cc. serum*	*m.-eq. per l. serum*	*m.-eq. per l. cells*	*mg. per 100 cc. serum*	*mg. per 100 cc. serum*	*gm. per 100 cc. serum*	*gm. per 100 cc. serum*
1	Resting.	231*	20.00	0.427	948	724	55.9	7.46	45.9		0.7		2.8	9.6	153	101	14.90	25.5	1.002	6.10
	After walking 3 hrs. at 3.2 miles per hr.	750									0.8	+0.1								
	After walking 6⅓ hrs. at 3.2 miles per hr.	750	21.68	0.463	938	723	52.0	7.47	41.6	−1.9	0.9	+0.2	4.3	9.6	157	107	14.82	26.8	1.163	7.10
2	Resting.	231*	20.25	0.425	944	729	54.3	7.45	45.6		0.9		4.9		155	85				
	After running $2\frac{1}{10}$ hrs. at 6.4 miles per hr.	2320	21.71	0.457	932	705	44.7	7.39	37.9	−3.5	2.1	+1.2	5.9		159	103				
3	Resting.	231†	19.75	0.433			54.2	7.45	45.4		0.9		4.4							
	After running ¼ hr. at 6.4 miles per hr.	2050	20.87								1.5	+0.6								
	After running 2 hrs. at 6.4 miles per hr.	2390	21.70	0.458	934	714	50.0	7.41	40.9	−2.1	1.8	+0.9	5.9	10.1	164			27.3	1.195	7.30
4	Resting.	231*	20.12	0.440			55.2	7.47	45.5		0.9		3.7							
	After running $2\frac{1}{10}$ hrs. at 6.4 miles per hr.	2280	21.50	0.458	930	722	51.0	7.43	42.4	−1.4	1.6	+0.7	5.1	10.2	164	116	15.31	27.7	1.220	7.45

5	Resting.	231*	20.00	0.433			54.5	7.45	44.6		0.7		5.0							
	After running $\frac{1}{10}$ hr. at 6.4 miles per hr.	2000	20.31								1.0	+0.3								
	After running $2\frac{1}{60}$ hrs. at 6.4 miles per hr.	2350	20.99	0.455			52.9	7.44	41.6	−1.3	1.2	+0.5	9.8							
6	Resting.	231*	19.60	0.438			52.0		43.6				3.5		157	113				
	After running $\frac{18}{60}$ hrs. at 9.8 miles per hr.	3100	21.04	0.467	932	727	30.2		25.7	−8.0	8.2	+7.0	6.1		163		14.90	30.6	1.220	7.44
	$\frac{1}{4}$ hr. after running stopped.	325	20.11						36.1	−3.2	2.9	+1.7								

* Assumed.

† Determined.

quotient to drop in the last half hour of work as it did in the other bouts.

Some of the variations in results may be attributed to training. At the outset the subject was untrained, except by an occasional game of squash. Later the body undoubtedly made a better adjustment to work than at the outset of the experiment. The effect of training seems to be reflected in changes of the blood, for, in comparing the four experiments with the same grade of work, it is evident that the composition of blood during the working state comes to resemble more and more closely that of the blood of the resting state in respect to oxygen capacity, cell volume, lactic acid concentration, and bicarbonate content. No significant change in oxygen consumption or carbon dioxide elimination can be attributed to training.

The average increase for all these experiments of the oxygen capacity was 7.5 per cent while the cell volume showed a 6.4 per cent increase (Table VII). In the change from rest to work, the fall in $(H_2O)_s$ was accompanied by a proportional increase in the protein of the serum. Cell water, serum water, serum protein, total fixed base, and total nitrogen did not vary greatly in the several bouts. Inorganic phosphate of serum always increased during work, whether or not phosphate had been previously ingested. This increase averaged 50 per cent, and was as great as 71 per cent in the last bout. Blood calcium, urea nitrogen, and non-protein nitrogen were not increased during work.

In Fig. 2 the values of r_H and r_{HCO_3}, determined in rest and work, are compared with the determinations of Van Slyke (13) for oxygenated horse blood. The values here given are calculated from determinations in oxygenated blood of cell water, serum water, cell volume, blood bicarbonate, and serum bicarbonate at several pressures of carbon dioxide. In calculating values of pH, $pK_s' = 6.12$ and $pK_c' = 5.93$ were used as the most probable values.

Observations were made on body weight before and after exercise. The average loss of weight in Experiments 2 to 6 was 2.1 kilos. If the quantity of carbon dioxide and water vapor excreted in the air is subtracted from this value, the remainder, approximately 1.6 kilos, must depend largely upon the weight lost by sweating. Burchardt (14) found the salt concentration of sweat

to be slightly less than 2 gm. per liter. This value being assumed, there was a loss of somewhat less than 3.4 gm. of chloride during each of these four experiments. This is of the same order as the diminished chloride excretion in the urine which amounted to 2 to 4 gm. per day for the bouts of work. Accompanying the increased loss of fluid as sweat there was a diminished excretion of water by the kidneys (Table VIII). The volume of urine excreted daily, gradually returned to normal 2 or 3 days after work.

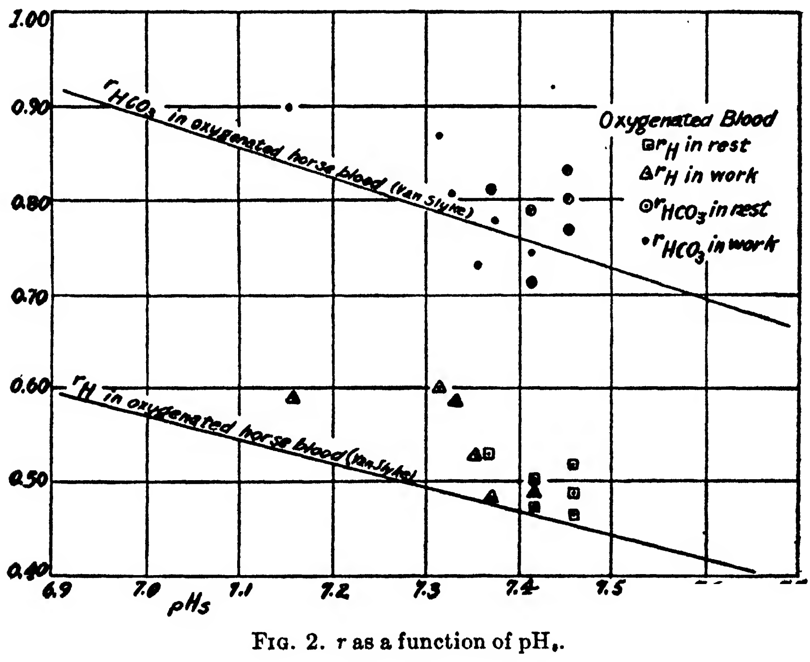

FIG. 2. r as a function of pH_s.

Table VIII clearly shows that nitrogen excretion was not independent of work. The values for the days of work vary little from those for days preceding work, but, on the days following work, there is uniformly a rise in nitrogen excretion. Thus the average for the 6 work days was 14.7 gm. while the average for the 1st day following the work day in the six experiments was 17.0 gm. This fact points to an increased destruction of protein during the bouts. The simplest explanation would be that it is

TABLE VIII.

Daily Observations on Urinary Constituents.

Time.	(1) Diuresis.	(2) pH	(3) Cl^-	(4) $SO_4^{=}$	(5) $HPO_4^{=} + H_2PO_4^-$ at observed pH.	(6) $HPO_4^{=} + H_2PO_4^-$ at pH = 7.4.	(7) Total acid at pH = 7.4 = (3) + (4) + (6).	(8) Fixed base.	(9) Ammonia N.	(10) Titratable acidity at pH = 7.4.	(11) Total base + titratable acidity = (8) + (9) + (10).	(12) Undetermined acids = (11) − (7).	(13) Total N.	(14) Urea N.	(15) Creatinine N.	(16) Creatine N.
days	*cc.*		*m.-eq.*	*m.-eq.*	*m.-eq.*	*m.-eq.*	*m.-eq.*	*m.-eq.*	*m.-eq.*	*m.-eq.*	*m.-eq.*	*m.-eq.*	*gm.*	*gm.*	*gm.*	*gm.*
1–5*	1180	5.5	115	73	36	62	250	190	41	50	281	31	15.67	13.44	0.63	0.05
6 (Experiment 1.)	940	5.5	146	68	33	58	272	228	43	46	317	45	15.17	13.36	0.63	0.06
7	1108	5.3	94	71	31	55	221	164	44	47	255	34	15.20	13.07	0.57	0.02
8	1645	5.6	134	84	41	70	289	208	68	54	333	41	16.88	15.01	0.67	0.02
9	1573	5.9	189	76	33	55	321	258	44	43	345	24	15.20	13.43	0.66	0.02
10	1270	5.2	218	60	28	50	329	276	48	45	369	40	14.47	12.21	0.66	0.02
11	1130	5.7	115	77	42	72	265	198	48	52	298	33	15.84	13.91	0.62	0.07
12	1083	5.1	95	80	36	63	238	163	46	52	261	23	14.94	13.11	0.70	0.02
13	1200	5.3	85	75	53	93	253	190	54	67	311	58	15.61	14.04	0.68	0.00
14 (Experiment 2.)	805	5.2	69	90	50	89	258	161	54	70	285	27	15.74	13.60	0.63	0.06
15	993	5.6	78	71	61	104	245	159	64	74	297	52	17.56	16.10	0.62	0.07
16 (Experiment 3.)	660	5.7	76	65	65	110	251	181	40	73	294	43	13.74	11.98	0.57	0.12
17	1138	5.7	117	72	38	65	254	198	43	47	288	34	16.90	15.01	0.67	0.04
18	1710	5.8	203	66	40	67	336	306	42	45	393	57	14.98	13.49	0.65	0.05
19	1510	5.7	140	91	32	54	285	213	48	48	309	24	14.11	12.61	0.65	0.04
20 (Experiment 4.)	690	5.6	72	75	33	57	204	155	49	48	252	48	14.39	12.57	0.68	0.01
21	1382	5.7	110	72	40	67	260	193	55	50	298	38	19.12	16.58	0.68	0.06
22	1630	6.1	142	76	64	102	320	251	48	64	363	43	16.03	14.75	0.67	0.05

23 (Experiment 5.)	735	5.6	49	75	80	136	260	178	40	87	305	35	15.18	14.11	0.67	0.07
24	1155	5.7	120	68	54	92	280	213	44	64	321	41	16.68	14.78	0.66	0.06
25	1300	5.6	136	71	65	111	328	243	46	73	362	34	15.14	13.39	0.67	0.04
26	2075	5.7	198	80	42	71	359	289	43	50	382	23	14.56	13.04	0.68	0.04
27 (Experiment 6.)	860	5.6	155	69	30	52	276	223	42	47	312	36	14.50	12.41	0.68	0.01
28	1325	5.6	175	80	33	57	312	235	54	50	339	27	14.86	13.06	0.70	0.03
29	1560	5.8	202	63	31	52	317	274	46	42	362	45	14.35	12.68	0.68	0.01
30	1040	5.5	138	68	31	54	260	202	46	53	301	41	13.80	11.22	0.71	0.01

* Control period. Average of weighed samples for first 5 days.

due to additional wear and tear of the muscles rather than to the use of protein as a direct source of energy. But, even though protein was so used (an assumption for which we have no evidence), the energy involved would be a very small part of the total. Another observation should be mentioned as regards the daily nitrogen excretion. In the first 13 days of the period the nitrogen excretion averaged 15.5 gm. while that for the last 6 days was 14.5 gm. Possibly, therefore, there has occurred a slight retention of nitrogen as a result of muscle hypertrophy. The excretion of urea closely follows that of nitrogen and needs no further comment.

The daily output of creatinine, practically constant throughout the period, confirms the observations of Shaffer (15) and others. Ingestion of NaH_2PO_4 was followed by no appreciable increase in the daily excretion of creatinine. The urinary phosphates fluctuated with exercise and with the intake of additional phosphate. Since the phosphates of the urine are concerned with maintaining the acid-base balance, it is doubtful if their fluctuation should be attributed to a share in muscular contractions.

The values given for the acid-base constituents of the urine are in milli-equivalents. This facilitates a comparison between individual acids or bases and the total acids or bases. The chloride and sulfate values, as determined, are the same at the observed pH of the urine as well as at a pH of 7.4 or that of the blood. The phosphates, however, vary with the pH. They are present partly as the monobasic salt, BH_2PO_4 and partly as the dibasic salt, B_2HPO_4. The difference between the base bound by phosphate at pH 7.4 and the base bound by phosphate at the observed pH of the urine constitutes a part of the economy of base.

The sulfates show little change in the exercise periods while the chlorides, as previously noted, show considerable change. On the work days, the chloride output falls considerably, a decrease which is only partially reflected in the excretion of total acid and total base. The radical which makes up this deficit among the acids is phosphate, which showed an increased excretion on all the work days, irrespective of the added intake of phosphate previous to these experiments.

SUMMARY.

This paper reports experiments on muscular exercise by an untrained subject during a period of 1 month, meanwhile remaining on a constant diet.

BIBLIOGRAPHY.

1. Sherman, H. C., Chemistry of food and nutrition, New York, 3rd edition, 1926.
2. Smith, H. M., *Carnegie Institution of Washington, Pub. No. 309*, 1922.
3. Friedemann, T. E., Cotonio, M., and Shaffer, P. A., *J. Biol. Chem.*, 1927, lxxiii, 335.
4. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, lxvi, 375.
5. Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, 1925, lxiii, 461.
6. Fiske, C. H., *J. Biol. Chem.*, 1922, li, 55.
7. Van Slyke, D. D., *J. Biol. Chem.*, 1927, lxxiii, 695.
8. Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd edition, 1927.
9. Folin, O., Laboratory manual of biological chemistry, New York and London, 4th edition, 1926.
10. Fiske, C. H., *J. Biol. Chem.*, 1921, xlvii, 59.
11. Van Slyke, D. D., and Cullen, G. E., *J. Am. Med. Assn.*, 1914, lxii, 1558.
12. Dill, D. B., Bock, A. V., and Talbott, J. H., *J. Physiol.*, in press.
13. Van Slyke, D. D., Hastings, A. B., Murray, C. D., and Sendroy, J., Jr., *J. Biol. Chem.*, 1925, lxv, 701.
14. Burchardt, W., *Arch. ges. Physiol.*, 1926, ccxiv, 169.
15. Shaffer, P. A., *Am. J. Physiol.*, 1908, xxii, 445.

THE DETERMINATION OF THE GUANIDINE BASES IN URINE.

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There is much uncertainty concerning the importance of the simple guanidine bases as etiologic factors in pathologic conditions, which is in part due to inadequate methods of analysis. The methods previously employed for the determination of the guanidine bases in urine have been subjected to much criticism (Greenwald, 1924; White, 1926–27). These authors concluded that no method was at present available which is adequate in proving the existence of small amounts of these bases in biological fluids.

The colorimetric method described herein for the determination of the guanidine bases in urine has been worked out in an effort to provide a convenient method for the study of certain problems connected with these bases. The method gives good recoveries of the guanidine bases when they are added in small amounts to normal urine. Since this method will indicate the probable amount of these bases in urine, it should prove useful in any procedures designed to isolate and prove their presence in urine.

The reagent used to develop a color with the guanidine bases is an alkaline nitroprusside-ferricyanide solution, previously described by the author (1927), which is based on the observation made by Tiegs (1924) that a solution of sodium nitroprusside left in contact with air and sunlight for several days gave a marked color reaction with an alkaline solution of the guanidine bases. The reagent differs from the one described by Marston (1924) in that the potassium ferrocyanide and hydrogen peroxide used by him have been replaced with potassium ferricyanide. This reagent gives nearly twice as much color with the guanidine bases as does Marston's reagent. The reagent is made by mixing

equal volumes of 10 per cent solutions of sodium nitroprusside, potassium ferricyanide, and sodium hydroxide, and diluting this mixture with 3 volumes of water. After 30 minutes, the reagent is ready for use, the color being a pale orange-yellow. This mixed reagent will usually keep for several hours. If a marked turbidity develops, a fresh reagent should be made. 1 cc. is added to every 5 cc. of the solution to be tested.

Various guanidine compounds having one or more of the hydrogen atoms of guanidine substituted by other groups have been tested as to their ability to produce color with the reagent. Methylguanidine, glycocyamine, and arginine in which 1 hydrogen atom is substituted, *as*-dimethylguanidine, creatine, glycocyamidine, and diphenylguanidine[1] in which 2 hydrogen atoms are substituted, all give a color with the reagent. Triphenylguanidine[1] in which 3 hydrogen atoms are substituted fails to give a color development with the reagent. Creatinine gives a very slow color development which is probably due to the oxidation of creatinine by the reagent. This reagent is not specific for the guanidine bases but the reaction is also given by urea, alcohol, glycerol, and aldehydes. The only amino acids that give the reaction are arginine and histidine. Histidine gives a green color in dilutions up to 1 mg. per cc. and only gives a red color in more concentrated solutions, as does also histamine and urocanic acid. Tyrosine, tyramine, phenol, and acetone do not produce any color. Creatinine gives $\frac{1}{310}$ the color given by an equal weight of guanidine if the reading is made 5 to 10 minutes after adding the reagent. The color given by creatinine develops slowly and remains practically constant between 5 and 9 minutes after the reagent is added. Creatine gives one-ninth as much color as does guanidine, but by evaporation with acid creatine can be converted into creatinine without changing the guanidine. Uric acid, salts, and ammonia, in quantities present in urine, interfere with the color production given by the guanidine bases.

This paper is concerned with the determination of the three simple guanidine bases, *i.e.* guanidine, methylguanidine, and *as*-dimethylguanidine, and not with their derivatives. The color

[1] It was necessary to dissolve diphenyl- and triphenylguanidine in alcohol and dilute 1 cc. of the alcoholic solution with 9 cc. of water before performing the test.

given by these compounds varies somewhat—guanidine giving more of an orange-red, while methyl- and dimethylguanidine give more of a red and less orange. Although the color matching against guanidine as a standard of methyl- and dimethylguanidine is not perfect, they may be matched with a reasonable degree of accuracy. We find that the color given by methylguanidine is almost exactly equivalent to guanidine, but dimethylguanidine gives about three-fifths as much color, 1 mg. of dimethylguanidine being equivalent to only 0.58 mg. of guanidine. Although guanidine may be used as the standard for the determination of any of these bases in urine, a greater degree of accuracy could be attained in the study of the excretion of these bases after administration by use of the base administered as a standard. In reading the color value of unknown solutions, it is necessary to choose a guanidine standard which is as near the color value of the unknown as is possible, so that the colorimetric readings lie between 17 and 22 with the standard set at 20. This is especially necessary when the readings are made with standards containing less than 0.1 mg. per 10 cc. With these precautions, the errors are, as a rule, less than 5 per cent.

Because of the number of interfering substances present, no direct determination of guanidine in the urine is possible. Adsorbents for these bases were tried and norit and blood charcoal offered the greatest promise. Norit, if used without first being washed with acid, gives a flocculent precipitate on addition of the reagent, which interferes with the determination of these bases, especially when present in small concentration. In our work, we have used only acid-washed norit prepared by suspending 200 gm. of norit in 2 liters of distilled water and adding 25 to 30 cc. of concentrated hydrochloric acid. The mixture is warmed to about 80° and filtered with suction on a Buchner funnel. The charcoal is washed on the funnel with another 2 liters of water and then dried for 24 hours at 110°. This acid-washed norit has been chosen in preference to blood charcoal because the guanidine value obtained from normal urine is 50 to 100 per cent less when norit is used than with blood charcoal, although the recovery of added guanidine is the same with both. Norit adsorbs the bases from alkaline solution. If the norit is treated with an acid solution of water or alcohol, the bases are set free and can then be deter-

mined in the filtrate. The determination of water solutions by this method gives recoveries averaging 90 per cent. Creatinine and creatine are also adsorbed, but do not interfere with the adsorption of the bases. Since creatine may be readily converted into creatinine, for which a correction is easily made, the adsorption does not interfere with the determination. When normal urine is treated with norit by the method described below, the nitrogen contained in the extract from this charcoal is only 4 to 7 per cent of the total nitrogen of the urine. 50 to 70 per cent of this nitrogen is contributed by creatinine. The extract does not contain an appreciable quantity of uric acid but does contain phenol and probably some imidazoles. The extract will be investigated further, especially with regard to the probable differences in the substances extracted by blood charcoal and norit and the identification of the substance responsible for the color produced in normal urine.

Unfortunately this method cannot be used to isolate methylguanidine from normal urine, since the treatment of creatinine with norit or blood charcoal produces methylguanidine in very small amounts. This was first brought to our attention by the fact that if creatinine solutions were treated with norit, the color produced by the extract from this charcoal was approximately 3 times greater than that produced by creatinine in the original solution. Methylguanidine has been isolated from such an extract and is responsible for at least a part of this increase in color production.

We feel, however, that this fact does not render the application of this procedure useless in studying the guanidine content of urine. First, the conversion of creatinine by norit to methylguanidine or rather the increase in color production is proportional to the creatinine concentration of the solution treated with norit. Second, although the conversion increases with the length of time the charcoal is in contact with creatinine, the conversion is practically constant under the conditions which we use for urine. Third, there is no reason to assume that the creatinine of the urine of different individuals would react differently. For these reasons, we believe that a correction can be made for creatinine which includes the increase in color due to norit treatment. Table I shows the increase of color given by creatinine on treat-

ment with norit. Creatinine, when added to urine, gives an increase in color value of the same magnitude as it does in water solution (Table II). The correction to be applied for the creatinine content of urine is approximately 0.7 mg. per 100 mg. of creatinine.

In making a determination of the guanidine content of the blood, such corrections are unnecessary, since the amount of

TABLE I.

Effect of Charcoal on Color Production Given by Creatinine.

Creatinine extracted.	Adsorbent.	Creatinine in extracts.	Guanidine value of color produced.	Guanidine value of color produced by 100 mg. creatinine.	Remarks.
mg.		*mg.*	*mg.*	*mg.*	
					25 cc. volume extracted in each case.
50	Blood charcoal.	34	0.29	0.58	
25	" "	16	0.16	0.64	
50	Norit.	24	0.31	0.62	
25	"	15	0.17	0.68	
20	"	15.7	0.11	0.55	Extracted at 4°.
20	"	11	0.19	0.95	" " 50°.
20	"	14	0.13	0.65	Room temperature 25°.
20	"	6.5	0.30	1.50	Stood 1 hr. with norit and 1 cc. 10 per cent NaOH.
20	"	13	0.135	0.68	Stood 1 hr. with 1 cc. 10 per cent NaOH before addition of norit.
15	"	10	0.11	0.74	
10	"	8	0.068	0.68	
5	"	3.5	0.036	0.72	

creatinine in the blood, even in advanced chronic nephritis, is so small that it produces no interference. Attention has been previously called to this observation (Major and Weber, 1927).

Normal human urine gives a color value equivalent to an excretion of 10 to 20 mg. of guanidine per 24 hours. Rabbit urine gives a value equivalent to 6 to 10 mg. per 24 hours, and dog

urine, figures of 10 to 30 mg. We cannot at present state what substance is responsible for this color value. The recoveries of added guanidine bases are the same for any type of urine. Apparently no substance is extracted, except creatinine, which interferes with the color production given by guanidine. Urine on standing gradually develops substances, perhaps due to creatinine decomposition, which give color production with the reagent sometimes amounting to 100 per cent increase. For that reason,

TABLE II.

Increase in Color Production Given by Urine Due to Addition of Creatinine.

Creatinine in urine per 100 cc.	Added creatinine per 100 cc.	Color value as guanidine per 100 cc.	Differences in guanidine value for added creatinine.	Additional color due to 100 mg. added creatinine.	Remarks.
mg.	*mg.*	*mg.*	*mg.*	*mg.*	
					Norit used in all experiments.
59		1.34			
	50	1.68	0.34	0.68	
63		1.68			
	100	2.26	0.58	0.58	
54		1.43			
	50	1.72	0.29	0.58	
63		1.70			
	50	2.03	0.33	0.66	
77		2.19			
	50	2.50	0.31	0.62	
48		1.21			
	50	1.49	0.28	0.56	

analysis should be made as soon as possible. On evaporation of the alcoholic extract from urine, a light chocolate-brown residue is obtained, which may contribute some of the color produced on addition of the reagent. The urine from one individual does not appear to vary greatly in the content of the compounds producing this darkening, nor do duplicates of the same urine vary. However, differences obtained in different individuals may be due to variations in the darkening of the residue and this must be taken into account. We have not been able to overcome

this difficulty, but feel that with proper care in the use of this method and with an appreciation of its shortcomings, it will still

TABLE III.

Recoveries of Guanidine Added to Pooled Urine.

Guanidine added per 100 cc.	Guanidine found per 100 cc.	Added guanidine recovered.		Remarks.
mg.	*mg.*	*mg.*	*per cent*	
0	2.27			Human Urine 1, 108 mg. creatinine per 100 cc.
2	4.01	1.74	87	Guanidine added as guanidine base.
3	4.88	2.61	87	
5	6.5	4.23	84.6	
7	8.23	5.96	85.1	
10	10.68	8.41	84.1	
0	2.30			50 mg. creatine added per 100 cc.
3	4.86	2.59	86.3	50 " " " " 100 "
5	6.53	4.26	85.2	50 " " " " 100 "
0	2.91			Human Urine 2, 120 mg. creatinine per 100 cc.
3	5.52	2.61	87	Guanidine base added as methylguanidine.
6	8.07	5.16	86	
12	12.98	10.07	83.9	
0	5.14			Human Urine 3, 145 mg. creatinine per 100 cc.
3	8.50	3.36	112	Guanidine base added as dimethylguanidine.
6	10.84	5.70	95	
10	15.00	9.86	98.6	
0	3.36			Rabbit urine, 77 mg. creatinine per 100 cc.
4	6.60	3.24	81	Guanidine added as guanidine base.
4	6.80	3.44	86	" base added as methylguanidine.
0	3.07			Dog urine, 50 mg. creatinine per 100 cc.
4	6.73	3.66	91.5	Guanidine added as guanidine base.
4	6.73	3.66	91.5	" base added as methylguanidine.
Average...........			88.9	

be useful in studying problems connected with the guanidine bases.

The method applied to urine is as follows: To 25 cc. of urine in a 50 cc. centrifuge tube add 10 cc. of 40 per cent lead acetate and 5 cc. of 10 per cent sodium hydroxide. The mixture is shaken and, after about 30 minutes standing, is centrifuged. The clear supernatant liquid may usually be pipetted off without filtering; 20 cc. of this supernatant liquid are pipetted into another centrifuge tube and 10 cc. of saturated disodium phosphate added and mixed. The precipitate of lead phosphate is removed by centrifuging and the clear supernatant liquid may be easily decanted. 25 cc. are extracted by the addition of 1 cc. of 10 per cent sodium hydroxide and 2 gm. of acid-washed norit, the norit being brought into suspension by shaking. The mixture is filtered immediately through an asbestos mat on a Gooch crucible. The charcoal is washed with two 10 cc. portions of water made alkaline by the addition of 2 or 3 drops of 10 per cent sodium hydroxide. The charcoal is then washed with 10 cc. portions of alcohol containing 6 cc. of N HCl per 100 cc., the filtrate being caught in a test-tube. 40 cc. of alcohol are used and this filtrate, after the addition of 5 cc. of 5 N hydrochloric acid, is evaporated to dryness on a water bath. The residue is dissolved in 20 cc. of water and 5 to 10 cc. are taken for the colorimetric determination.

The standards usually utilized are 0.07, 0.1, 0.15, and 0.2 mg. of guanidine[2] per 10 cc. and 2 cc. of the reagent added. The unknowns are made to the same volume and 2 cc. of the reagent added. The readings are made after 5 minutes and must be completed 9 minutes after the addition of the reagent.

The calculation of the guanidine content of urine, if one-half of the residue is taken for analysis, is as follows:

$$\frac{20}{\text{Reading of unknown}} \times \text{mg. guanidine in standard} \times 19.2 \text{ mg. guanidine per 100 cc. urine.}$$

This must be multiplied by 2 if one-fourth of the residue is used. The time required to complete a determination is about 2 hours.

[2] A stock solution of guanidine carbonate containing 1 mg. of guanidine per cc. is made by dissolving 0.1524 gm. of guanidine carbonate in 100 cc. of 0.1 N HCl. The standard solution containing 0.02 mg. of guanidine per cc. is made by diluting 5 cc. of the stock solution to 250 cc. The solutions are preserved with chloroform.

Table III shows recoveries with human, dog, and rabbit urine of added guanidine.

SUMMARY.

A method for determining guanidine in urine is described which shows a high percentage of recovery when guanidine is added to normal urine. This method should prove useful in studying the fate of these compounds when they are introduced into the body. Normal urine gives a color with this reagent, but we do not yet know what substance is responsible for this color production.

BIBLIOGRAPHY.

Greenwald, I., *J. Biol. Chem.*, 1924, lix, 329.
Major, R. H., and Weber, C. J., *Arch. Int. Med.*, 1927, xl, 891.
Marston, H. R., *Australian J. Exp. Biol. and Med. Sc.*, 1924, i, 99.
Tiegs, O. W., *Australian J. Exp. Biol. and Med. Sc.*, 1924, i, 93.
Weber, C. J., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 712.
White, F. D., *J. Biol. Chem.*, 1926–27, lxxi, 419.

THE PREPARATION OF *d*-ARGININE MONO-HYDROCHLORIDE.

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INTRODUCTION.

Kossel and Gross (1923, 1924) have introduced a method of preparation of arginine carbonate from hydrolyzed proteins by an initial isolation of the arginine as a salt of flavianic acid (1-naphthol-2, 4-dinitro-7-sulfonic acid). In their first paper they favored the use of concentrated hydrochloric acid to effect the hydrolysis of the protein; in their second paper they advised sulfuric acid. The latter method requires the removal of the acid by means of calcium or barium hydroxide and involves loss of time or material, depending on the thoroughness of washing of the precipitate. Hydrochloric acid may be removed by distillation and subsequent neutralization of the remaining free acid.

From the arginine flavianate, Kossel and Gross prepared the carbonate by two procedures. For this purpose arginine flavianate may be decomposed by heating with 33 per cent sulfuric acid, the free sulfonic acid filtered off, the sulfonic acid removed with an excess of barium hydroxide, the barium precipitated as the carbonate, and arginine carbonate obtained by evaporation to dryness. The second method involves the use of barium hydroxide to remove the flavianic acid from the arginine salt dissolved in an excess of ammonium hydroxide. After the removal of the barium, the carbonate is prepared as above. The authors advised purifying the arginine by recrystallization of the picrate before final conversion to the carbonate.

Pratt (1926) has offered a modification of the method of Kossel and Gross. He reverts to the use of hydrochloric acid to hydrolyze the protein, recrystallizes the arginine flavianate from dilute sulfuric acid in an attempt to insure the purity of the final product, and decomposes the flavianate by solution in hot dilute sulfuric acid and extraction of the free sulfonic acid with *n*-butyl alcohol. Arginine carbonate is not a satisfactory final product because of the uncertainty of its composition and purity. Pratt tests for the purity of his carbonate by preparing pure arginine picrolonate from it. Obviously, such a test is not satisfactory as the pure derivative might readily be obtained in the presence of numerous impurities.

Vickery and Leavenworth (1927, *a*) prefer to prepare arginine by a modifi-

cation of the Kossel and Kutscher (1900) procedure using silver oxide as the precipitant. They obtain and purify the arginine as the picrate, finding the latter procedure more convenient than decomposition of arginine flavianate. Their method requires the filtration and washing of barium sulfate and, for economy, the recovery of the silver. Both of these operations are cumbersome and time-consuming.

To avoid the difficulties of filtration and recovery of reagents involved in the methods cited above and to secure a stable, pure final product, a modification of the flavianic acid method of Kossel and Gross has been devised. Hydrolysis of the protein and decomposition of the arginine flavianate is accomplished by means of hydrochloric acid. Arginine monohydrochloride is obtained as the final product and can be efficiently purified by precipitation with alcohol from concentrated aqueous solutions.

In the preparation of arginine monohydrochloride from solutions containing an excess of hydrochloric acid, use has been made of the convenient basicity of aniline, and the solubility of aniline hydrochloride in alcohol. The basic dissociation constants as given by Michaelis (1922) are:

Aniline.	$K_b = 4.6 \times 10^{-10}$
Arginine.	$K_{b_1} = 1.0 \times 10^{-7}$
	$K_{b_2} = 2.2 \times 10^{-12}$

Aniline, therefore, combines with all the hydrochloric acid in excess of that united with the stronger basic group of arginine.

EXPERIMENTAL.

Hydrolyze 1 kilo of a good grade of gelatin by vigorous boiling for 24 hours in a 12 liter flask with 2300 cc. of C.P. concentrated hydrochloric acid. Concentrate to a thick sirup under reduced pressure. Dissolve the residue in about 3000 cc. of water, filter, and neutralize to Congo red with a concentrated solution of sodium hydroxide. Precipitate the arginine with 300 gm. of flavianic acid in 1500 cc. of water. During the addition of the precipitant, and for about an hour thereafter, the mixture should be vigorously stirred in order to prevent the formation of crusts. Set the flask in an ice box for several days to complete the precipitation.

Filter off the arginine flavianate and wash it with ice water until the washings are entirely free of chloride. Thoroughly dry the

precipitate in the air or in an oven at about 100°. Transfer the flavianate to a 2 liter flask and for every 100 gm. add 200 cc. of concentrated hydrochloric acid. Heat the mixture on a steam cone for 1 to 2 hours with frequent stirring or shaking. Cool in an ice bath, and filter off the free flavianic acid on a filtros plate sealed into a 15 cm. Buchner funnel with paraffin. The filtration may be done on a porous glass filter. If the filtrate is not clear, pour it back through the filter. Wash the precipitate with cold concentrated hydrochloric acid until the washings have only a light yellow color. Concentrate the filtrate and washings *in vacuo* to a thick sirup, preventing foaming with a few drops of caprylic alcohol. Dissolve the sirup by warming with 400 cc. of 95 per cent alcohol and place the solution in the ice box overnight for any flavianic acid to separate as the arginine salt.

Filter off the small amount of flavianate, add 50 gm. of aniline to the filtrate, stir, and scratch the flask vigorously with a glass rod until the arginine monohydrochloride is well crystallized. Placed it in the ice box overnight. Filter off the arginine monohydrochloride and wash with alcohol until the washings are only slightly colored.

Dissolve the arginine monohydrochloride in 300 to 500 cc. of water and decolorize it with about 10 to 15 gm. of norit. Evaporate the filtrate *in vacuo* in a 2 liter round-bottom flask to a thin sirup. Cool to room temperature and add 95 per cent alcohol, with stirring, until a permanent turbidity appears. Scratch the sides of the flask until crystallization starts, and then add slowly 400 cc. of alcohol. Set the flask in the ice box overnight. Filter off the precipitate and wash several times with alcohol. 75 to 80 gm. of arginine monohydrochloride, melting at 216–217° (uncorrected), are obtained.

DISCUSSION.

Attempts to use a low grade of gelatin, such as glue, have necessitated recrystallization of the arginine flavianate, and have resulted in lower yields of arginine monohydrochloride. The gelatin that gave most satisfactory results was an ordinary commercial product having 16.85 per cent of total nitrogen.[1]

[1] Gelatin from the Wilson Laboratories labeled "Free of sulfur dioxide and other preservatives" and "Bacto-Gelatine" of the Digestive Ferments Company were the only samples of purified gelatin used. Any other brands of purified gelatin should give as good results.

Following hydrolysis, if the free hydrochloric acid remaining after the distillation is not neutralized, a mixture of the mono- and the diflavianates of arginine is obtained. Some arginine, therefore, is not precipitated unless more flavianic acid is added. Vickery and Leavenworth (1927, *b*) have obtained a yellow product which they regard as the diflavianate, $C_{26}H_{26}N_8S_2O_{18}$, and have noted its ready transformation to the orange monoflavianate, $C_{16}H_{20}N_6SO_{10}$, by treatment with water. Their observations have been confirmed. Some of the diflavianate has been prepared, recrystallized from 4 per cent hydrochloric acid, and analyzed with the following results:

	Found.	Calculated for:	
		$C_{26}H_{26}N_8S_2O_{18}$	$C_{16}H_{20}N_6SO_{10}$
	per cent	*per cent*	*per cent*
C........................	38.42	38.88	39.32
H........................	3.65	3.27	4.12
S........................	7.90	7.99	6.56

Further confirmation of the identity of the yellow compound was obtained by treating some of the supposed arginine diflavianate with an excess of arginine monohydrochloride and securing a yield of 97.8 per cent of the theory of arginine monoflavianate. Also, on boiling a sample of the diflavianate with water, cooling, and filtering, 96.3 per cent of the theory of arginine monoflavianate was obtained. The diflavianate melts with decomposition at 170–175° (uncorrected) when rapidly heated or plunged into a bath at that temperature. If heated slowly, considerable shrinkage in volume occurs at about 160°, and melting with decomposition at about 245°.

In place of the free flavianic acid, the sodium salt[2] may be used for the precipitation of the arginine, if an amount of hydrochloric acid equivalent to the sodium be left in the solution, or added to the solution of the sodium flavianate before addition to the hydrolysate. The flavianic acid recovered from the decomposition of the arginine flavianate, needs no treatment for use other than exposure to the air for several days to remove the hydrochloric acid.

[2] The "Certified" Naphthol Yellow S of the National Aniline and Chemical Company, Inc., gives excellent precipitates of arginine flavianate. Their technical grade contains a considerable amount of impurities and can only be used successfully after conversion to the free acid.

The properties of the arginine monohydrochloride as obtained above are as follows:

	Found.	Calculated for $C_6H_{14}N_4O_2 \cdot HCl$.
	per cent	*per cent*
Total N	26.52	26.64
Amino "	6.68	6.66
Chlorine	16.76	16.83

There is no water of crystallization in the preparations obtained by precipitation with alcohol, nor do samples recrystallized from water show any loss in weight when heated overnight in an oven at 110°. Schulze and Steiger (1887) did not find water of crystallization in their arginine monohydrochloride. Gulewitsch (1899) and Hedin (1895–96) found 1 molecule, and Lawrow (1899) reports ½ molecule of water of crystallization. The corrected melting point of arginine monohydrochloride is 222° in a capillary tube. The melting point is not changed by recrystallization from water. Gulewitsch (1899) records a melting point of 208–209°.

BIBLIOGRAPHY.

Gulewitsch, W., *Z. physiol. Chem.*, 1899, xxvii, 178.

Hedin, S. G., *Z. physiol. Chem.*, 1895–96, xxi, 155.

Kossel, A., and Gross, R. E., *Sitzungsber. Heidelberg Akad. Wissensch., Abt. B*, 1923, i, 1, abstracted in *Chem. Abst.*, 1924, xviii, 1681; *Z. physiol. Chem.*, 1924, cxxxv, 167.

Kossel, A., and Kutscher, F., *Z. physiol. Chem.*, 1900, xxxi, 165.

Lawrow, D., *Z. physiol. Chem.*, 1899, xxviii, 388.

Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 2nd edition, 1922, pt. 1, 34, 60.

Pratt, A. E., *J. Biol. Chem.*, 1926, lxvii, 351.

Schulze, E., and Steiger, E., *Z. physiol. Chem.*, 1887, xi, 43.

Vickery, H. B., and Leavenworth, C. S., *J. Biol. Chem.*, 1927, *a*, lxxv, 115; 1927, *b*, lxxii, 403.

THE CONDUCTIVITY METHOD AND PROTEOLYSIS.

II. AN INTERPRETATION OF THE CONDUCTIVITY CHANGES.*

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(Received for publication, May 22, 1928.)

In the first paper of this series (1), it was shown that the conductivity changes taking place during digestion of egg albumin by pepsin could be satisfactorily accounted for on the basis of the peptone produced. It was noted that because of the variable effect of peptone on the conductivity at various pH levels, the conductivity method was not suited to the determination of optimum pH of peptic action. Northrop (2) has pointed out an additional disadvantage of the method, which, if true, will greatly curtail its usefulness. He found that the conductivity curves of digestion did not parallel the amino nitrogen curves. He attributed this discrepancy between the two methods to the antagonistic effect of the carboxyl and amino groups on the conductivity, but since he published no data on this point, it seemed advisable to repeat the experiments.

Pepsin Digestion.

Accordingly, solutions of egg white were diluted to 3 per cent strength with HCl solutions of various concentrations as described in the previous paper. 25 cc. were used in the conductivity cells with 1 cc. of a 4 per cent scale pepsin solution (1:3000 U.S.P.). For the amino nitrogen determinations, 200 cc. of the same 3 per cent egg white solution in a flask were mixed with 8 cc. of the pepsin solution. The conductivity and amino nitrogen were determined

* This paper was submitted in partial requirement for the degree of Doctor of Philosophy. The work was carried out under the direction of Dr. H. C. Bradley to whom the author wishes to express his appreciation.

from time to time for about 24 hours. The results of two such experiments at different pH levels are shown in Figs. 1 and 2. At pH values between those given, the curves show a gradual conver-

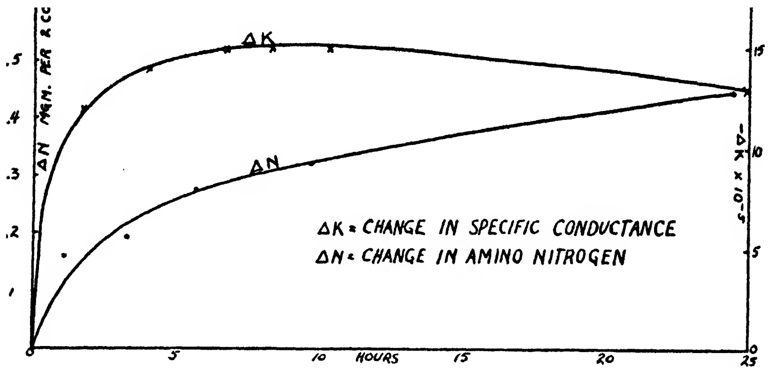

FIG. 1. Pepsin digest. pH 2.95.

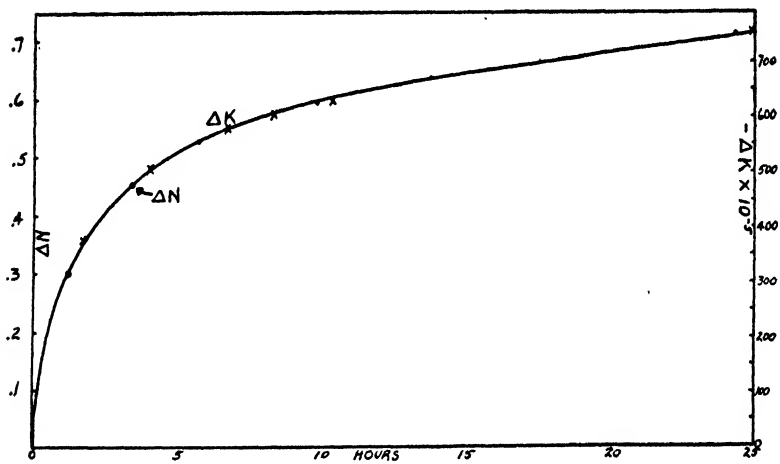

FIG. 2. Pepsin digest. pH 1.38.

gence. At pH 1.15, the two curves are still coincident, and the rate of change in conductivity is greatest. Results of a similar experiment at pH 3.30 were not entirely satisfactory due to the slight activity of pepsin at this acidity. They do, however, indi-

cate that the time course is different than that for the amino nitrogen change, and the conductivity increased, whereas it decreased in all the others. Northrop's statement that the greatest changes in conductivity occur at pH 2.6 was not confirmed. His statement that the conductivity and amino nitrogen curves follow a different time course must be modified with reference to the pH, since the two curves are coincident when the pH is below 1.38. It appears that the maximum change in both ΔK and ΔN occur at the same pH. This modifies our earlier statement that the conductivity method was not applicable for determining the

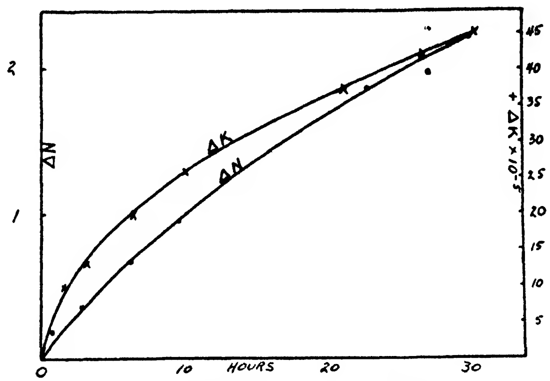

FIG. 3. Papain digest. pH 6.82.

optimum pH for peptic digestion. It is a curious fact that this maximum also coincides with the maximum binding power of peptone for HCl, as was shown in the first paper (1). We shall discuss this later.

Papain Digestion.

In order to extend the study of these changes over a wider pH range, we have also used papain activated with HCN, which has been shown (3) to resemble pepsin in its catalytic behavior, but is active at a lower hydrogen ion concentration. Both pepsin and papain are believed to split peptide linkages, and thus give rise to amino and carboxyl groups. Papain, however, carries the cleavage further than pepsin, presumably because of the presence of a peptidase in the preparation; but the early stages of protein

cleavage at least appear to be similar in character to peptic digestion. The results are shown in Figs. 3 and 4.

Summarizing these papain experiments briefly then, we find that when the pH is below 7.36, the conductivity changes do not follow the nitrogen changes, but above this pH the two curves are again coincident. These results are in agreement with those of Bayliss (4) who showed that the conductivity curves in a tryptic digest (pH about 8.0) were parallel to the curves of increase of soluble nitrogen in a tannic acid filtrate. This latter method of following proteolysis gives essentially the same picture of a tryptic digest as the Van Slyke method does. We have confirmed our former observation that below pH 3.00 the conductivity always decreases, but above this figure it always increases.

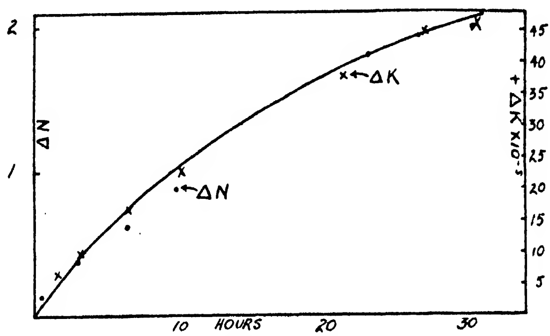

FIG. 4. Papain digest. pH 7.36.

Changes in pH and pCl during Digestion.

In order to interpret these curves, we must be able to follow the changes in concentration of each of the ionic species present. Practically, this resolves itself into a measurement of the changes in hydrogen and chloride ions, since we have no direct method of measuring the ionic concentration of the protein derivatives. Accordingly, pepsin and papain digestions were followed by means of the hydrogen electrode. The results are given in Table I.

The pCl was determined on the pepsin digests only, since it was thought that the presence of HCN in the papain digest would interfere with the action of the AgCl electrode. The measurements made on peptic digestion confirm the work of Rohonyi (5)

that the pCl is practically constant. Hitchcock (6) found that proteins did not bind Cl^-, and Ringer (7) showed the same for albumoses. We may, therefore, for the present at least, assume that the changes in pCl which occur during digestion are insignificant when compared with the changes in H^+.

If only H^+ is bound or liberated during digestion, we should be able to calculate the changes in conductivity which occur in the relation:

$$\Delta K \quad \frac{\Delta H^+ \times \Lambda_{H^+}}{1000}$$

where ΔK is the change in specific conductivity, ΔH^+ the change in H^+ concentration expressed as milli-equivalents per cc., and

TABLE I.

Calculation of ΔK from pH Changes in Digests.

	ΔH^+	ΔK calculated.	ΔK observed.
Conductivity decreasing and H^+ bound.	1.75×10^{-2}	7.08×10^{-3}	6.71×10^{-3}
	1.233×10^{-2}	4.99×10^{-3}	2.85×10^{-3}
	1.98×10^{-3}	8.10×10^{-4}	6.50×10^{-4}
	3.88×10^{-4}	1.20×10^{-4}	4.00×10^{-5}
	1.14×10^{-3}	4.62×10^{-4}	2.00×10^{-4}
Conductivity increasing and H^+ liberated.	1.11×10^{-6}	4.50×10^{-7}	4.50×10^{-4}
	6.87×10^{-7}	2.80×10^{-7}	4.60×10^{-4}
	5.57×10^{-7}	2.20×10^{-7}	4.60×10^{-4}

Λ_{H^+} is the ionic conductance of the hydrogen ion which has the value 405 at 37°.

Table I shows that when the conductivity is decreasing, the calculated value of ΔK is greater than the observed, whereas the reverse is true when the conductivity increases. Rohonyi explained this discrepancy on the basis of the effect of the peptone ions in increasing the conductivity. We have followed this suggestion and have studied the effect of various protein materials on the conductivity, noting also their influence on the pH. From the amount of hydrogen bound, we have calculated the effect on the conductivity with the equation given above. Table II shows the results when glycine was used. We see that in this case the observed ΔK may be satisfactorily accounted for by the decrease

in hydrogen ions, the effect of the glycine ion being insignificant. Quite a different result was obtained with glutamic acid.

We are somewhat handicapped when using dicarboxylic acids because of their insolubility. However, we see from Table III that the calculated value of ΔK at low pH levels is always greater than the observed.

We have used in addition a commercial mixture of amino acids called aminoids and Witte's peptone, both of which contain

TABLE II.

Calculation of ΔK from pH Changes When Glycine Is Added to HCl.

C_{H^+} bound.	ΔK calculated.	ΔK observed.
0.0066	2.29×10^{-3}	2.00×10^{-3}
0.0122	4.23×10^{-3}	4.20×10^{-3}
0.0192	6.65×10^{-3}	7.70×10^{-3}
0.0314	1.088×10^{-2}	1.070×10^{-2}
0.0392	1.360×10^{-2}	1.320×10^{-2}

TABLE III.

Calculation of ΔK from pH Changes When Glutamic Acid Is Added to HCl.

C_{H^+} bound.	ΔK calculated.	ΔK observed.
0.0206	8.34×10^{-3}	2.30×10^{-3}
0.01437	5.82×10^{-3}	1.49×10^{-3}
0.004323	1.75×10^{-3}	8.48×10^{-4}
0.000488	$1.98^{*} \times 10^{-4}$	4.1×10^{-6}

* In this case we noted a very slow attainment of equilibrium; it took 3 days to reach the value given. Microscopical examination and the existing acidity eliminated the possibility of infection.

dicarboxylic acids and both show this discrepancy. Aspartic acid has been shown (1, 4) to increase the conductivity considerably. We, therefore, feel justified in tentatively attributing this discrepancy in the digest to the dicarboxylic acids present.

By a further study of this discrepancy during digestion, it may be possible to determine when these acids are liberated and whether they occur as amides as has been suggested (8).

Mechanism of Binding Hydrogen Ions.

Regarding the mechanism by which these substances change the hydrogen ion concentration, two views have been advanced. Mori (9) found that peptone bound hydrogen ions according to the Freundlich adsorption formula:

$$\log \frac{x}{m} = \log \alpha - \frac{1}{n} \text{pH}$$

where x is the concentration of hydrogen ions bound and m is the weight of peptone used. α and n are constants. Others have

TABLE IV.

Binding of H^+ by Glycine. Calculated from the Freundlich Adsorption Formula.

$\frac{x}{m}$ observed.	$\frac{x}{m}$ calculated.
1.862×10^{-2}	1.045×10^{-2}
4.09×10^{-3}	6.12×10^{-3}
4.14×10^{-3}	4.78×10^{-3}
7.27×10^{-3}	7.19×10^{-3}
4.84×10^{-3}	5.21×10^{-3}
3.41×10^{-3}	3.14×10^{-3}
2.42×10^{-3}	2.55×10^{-3}
7.56×10^{-3}	7.81×10^{-3}
5.61×10^{-3}	5.33×10^{-3}
4.405×10^{-3}	4.93×10^{-3}
3.885×10^{-3}	3.96×10^{-3}

held that the reaction may be satisfactorily described on the basis of the ionization of ampholytes. It is possible that these two views are not incompatible. Indeed it appears very probable that they are not when we apply the adsorption formula to the binding of hydrogen ions by glycine. Table IV shows that there is a very good agreement between the calculated and observed values for $\frac{x}{m}$. In the higher concentrations of acid, however, the agreement is not so good as in the lower.

Katsu (10) has shown that the adsorption formula also holds

for the binding of chloride ions by glycine, but in view of the fact that glycine also binds silver ions, these results with the Ag-AgCl electrode may need reinterpretation. We intend to evaluate this effect of binding silver ions and hope to report our

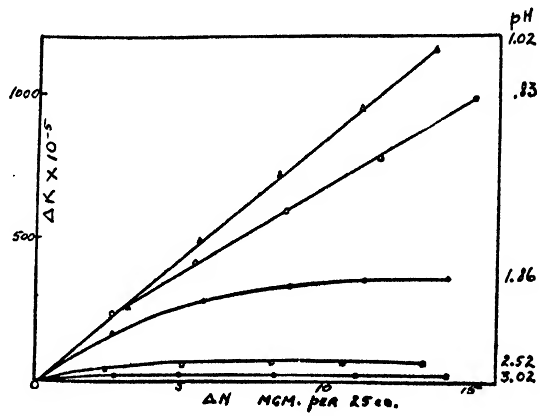

FIG. 5. Glycine in HCl.

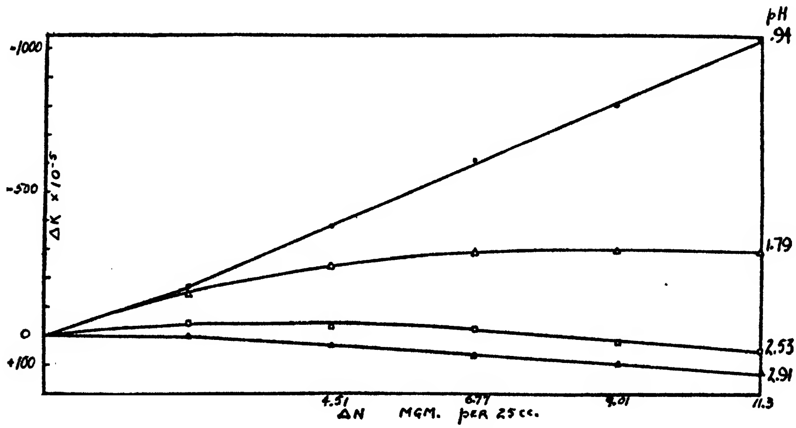

FIG. 6. Aminoids in HCl.

results later. It is of interest in this connection that Hitchcock (6) noted that AgCl is more soluble in protein solutions than in H_2O, but the effect was too small to consider.

It seems clear that this type of adsorption which glycine appar-

ently exhibits is a different sort from the type ordinarily considered where surfaces are concerned, for here we are dealing with a substance of molecular dimensions, certainly in true solution. As far as glycine is concerned, then, it seems reasonable to assume that the binding of hydrogen ions occurs according to the well known laws of chemical reaction, and the fact that the Freundlich formula also applies indicates that here, at least, it is merely a special case of the mass law (11).

But what are the conditions existing in the digests and in the case of peptone? May we not be dealing, in these cases, with adsorption on surfaces? If we are, then we would expect quite

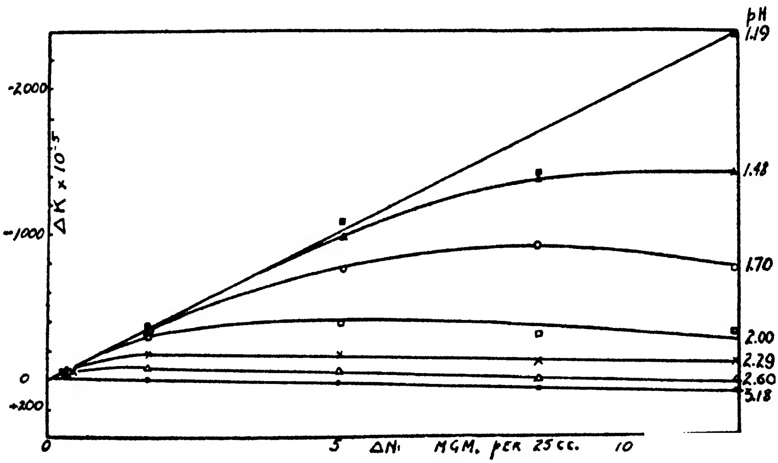

FIG. 7. Witte's peptone in HCl.

different relationships between the amino nitrogen and the hydrogen ions bound than we found in the case of glycine. Figs. 5 to 8 show the relation between the amino nitrogen and the conductivity. This is essentially the same as the relation between amino nitrogen and hydrogen ions bound. We see here a remarkable similarity in the type of curves obtained for all the preparations used and the digests. If this similarity is any indication of similarity of mechanism, then we must conclude that changes in hydrogen ion concentration during digestion are brought about by chemical combination with the products.

One of the striking facts brought out here is that there is a pH

at which there is a direct relationship between the amino nitrogen and the change in conductivity. Values above this give curves of conductivity which show a progressive falling off in slope. The complete meaning of this is not clear, but it seems likely that with decreasing acidity there is a conversion of the active groups present into an inactive form. On the basis of a few calculations it seems likely that the phenomenon concerned may be explained on the basis of Bjerrum's (12) theory of ampholytic dissociation. The

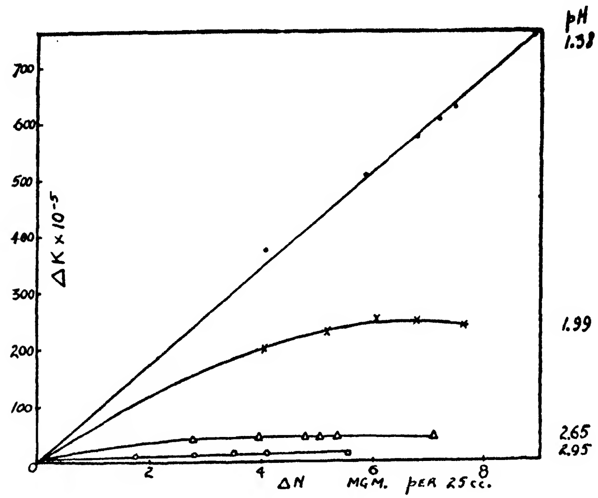

FIG. 8. Pepsin digests.

following tabulation gives the calculated value of ΔK as compared with the observed values with the equation of Bjerrum

$$\frac{{}^{+}H_3NCH_2COO^{-}}{{}^{+}H_3NCH_2COOH} = \frac{K_s}{H^{+}}$$

where $K_s = 10^{-2.33}$.

*Effect of Glycine on Conductivity of HCl.**

Calculated (Bjerrum).
2.05×10^{-3}
4.31×10^{-3}
7.66×10^{-3}
1.055×10^{-2}
1.285×10^{-2}

* For the observed values see Table II.

When the pH is 1.04 we find excellent agreement. At lower acidities, however, the calculated values are consistently lower than the observed.

DISCUSSION.

We see, then, that there are two regions in which the conductivity and the amino nitrogen methods agree in their picture of proteolysis. In the region between there is disagreement. Therefore, if one is interested merely in the progress of proteolysis, it is essential that the studies with the conductivity method be made in these regions of agreement. But why should there be two regions of agreement? This we believe is due to the fact that the materials concerned are ampholytes which ionize as acids at one pH and as bases at another. According to Bjerrum amino acids and peptides exist almost wholly as "Zwitterionen" represented as follows:

$$^{+}H_3N\ R\ COO^{-}$$

In acid solutions: $^{+}H_3N\ R\ COO^{-} + H^{+} = {}^{+}H_3N\ R\ COOH.$

In alkaline solutions: $^{+}H_3N\ R\ COO^{-} + OH^{-} = H_2N\ R\ COO^{-} + H_2O.$

The constants of acid and basic dissociation derived from these equations express the properties of the substances much better than the older ones did. They are related to the older constants by the following equations:

$$K_s = \text{new acid dissociation constant} = \frac{K\ \text{water}}{K_b\ \text{(old basic dissociation constant)}}.$$

$$K_B = \text{new basic dissociation constant} = \frac{K\ \text{water}}{K_a\ \text{(old acid dissociation constant)}}.$$

From the above equations, it can be seen that the "Zwitterionen" ($^{+}NH_3\,R\,COO^{-}$) cannot contribute to the conductivity since they are electrically neutral. But the other forms ($^{+}H_3N$-$R\ COOH$ and $H_2N\ R\ COO^{-}$) may influence the conductivity, the magnitude depending on the concentration of hydrogen or of hydroxyl ions respectively. A simple calculation will illustrate the relative effect of the acid dissociation constant on the conductivity. If we calculate the per cent of $^{+}NH_3\ R\ COOH$ for glycine

and aspartic acid at the same pH we note that the per cent of the glycine ion is greater than the corresponding figure for aspartic acid as the following tabulation shows.

Effect of K_s on Per Cent of Cations Present.

pH	$^+NH_3CH_2COOH$ $K_s = 2.33$	$HOOCCHNH_3^+ - CH_2COOH$ $K_s = 1.98$
	per cent	*per cent*
1.07	94.79	89.05

This means that for a given weight of amino nitrogen, glycine binds more hydrogen ions than aspartic acid at the same pH, and therefore the former can decrease the conductivity more than the latter. This is exactly what we should expect if the dicarboxylic acids are responsible for the discrepancy noted above.

It was also noted above that the optimum pH for peptic digestion coincided with the optimum pH for the binding of HCl. On the basis of Bjerrum's theory, these ampholytes have bound the maximum acid when they are most completely in the form ^+H_3N-R COOH. If this ion is the true substrate for pepsin (13), we would expect the maximum rate of digestion at the pH giving the greatest concentration of this ion.

We must leave the interpretation of the papain digests for a future paper.

SUMMARY.

1. It has been shown that on the pH scale there are two regions in which the conductivity changes and amino nitrogen changes are identical in albumin digests.

2. The changes in conductivity produced by addition of ampholytes to HCl solutions may be quantitatively accounted for in some cases by the change in hydrogen ions. In others, the effect of dicarboxylic acids must be considered also.

3. Doubt has been cast on the applicability of the Ag-AgCl electrode in chlorine ion determinations when protein materials are present, due to the binding of Ag ions.

4. The hydrogen ions are probably bound according to the mass law.

5. Bjerrum's theory of ampholytic dissociation seems to apply in at least one of the cases studied.

BIBLIOGRAPHY.

1. Baernstein, H. D., *J. Biol. Chem.*, 1927, lxxiv, 351.
2. Northrop, J. H., *J. Gen. Physiol.*, 1919, ii, 113.
3. Willstätter, R., and Grassmann, W., *Z. physiol. Chem.*, 1924, cxxxviii, 184.
4. Bayliss, W. M., *J. Physiol*, 1907–08, xxxvi, 221.
5. Rohonyi, H., *Biochem. Z.*, 1912, xliv, 165.
6. Hitchcock, D. I., *J. Gen. Physiol.*, 1922, v, 383.
7. Ringer, W. E., *Z. physiol. Chem.*, 1915, xcv, 195.
8. Schryver, S. B., The general characters of the proteins, London and New York, 1909, 34.
9. Mori, S., *J. Biochem.*, Japan, 1927, vii, 411.
10. Katsu, Y., *J. Biochem.*, Japan, 1927, ii, 151.
11. Robertson, T. B., *Z. Chem. u. Ind. Kolloid.*, 1908, iii, 49.
12. Bjerrum, N., *Z. physik. Chem.*, 1923, civ, 147.
13. Northrop, J. H., *J. Gen. physiol.*, 1920, iii, 226.

A METHOD FOR THE BIOLOGICAL ASSAY OF COD LIVER OIL.

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Standardization of cod liver oil for medicinal purposes was at first made with respect to vitamin A content (1) and growth-promoting properties (2). More recently, however, interest has centered in the vitamin D content of the oil, and biological tests are directed toward determinations of antirachitic potency. Methods for such a biological assay are indicated by the facts established in recent years during the course of investigation relative to rickets.

Of importance was the finding that a condition simulating human rickets could be established experimentally in rats and other animals by feeding certain deficient diets. Diets containing calcium and phosphorus in disproportionate amounts, particularly those of high calcium-low phosphorus type (3), gave rise to rachitic developments, provided that at the same time vitamin D was absent from the diet. Cod liver oil, which had been prescribed for years as a curative for rickets, was now shown experimentally to be particularly rich in the antirachitic factor which functions in the prevention or healing of rickets induced by an unfavorable dietary régime. Moreover, it was established that ultra-violet rays had the same effect as cod liver oil in preventing or curing rickets.

Histological study of the rachitic bone indicated that very definite changes took place even before gross lesions were distinctly

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evident. Not only did the rachitic bone present a picture quite different from the normal bone, but in addition, there was a rather definite sequence of changes to be noted as healing progressed. Observation of histological changes in the bone thus presented a method for following the course of an experimental rickets produced by faulty diet, or the progress of healing under the influence of the antirachitic factor.

Coincident with the changes in the structure of the bone there was found to be a change in the mineral content; particularly, the ash of rachitic bones fell far below the normal level only to increase again as calcification of healing took place (4–7). Since tricalcium phosphate constitutes the greater part of the inorganic matter of the bone it is to be expected that the calcium and phosphorus content of rachitic bones would be less than in the case of normal bones. That there might be a definite variation in these calcium and phosphorus values as rickets develops or as healing progresses is a possibility indicated by some of the experimental work with regard to the relationship (5, 8).

It was observed early in the course of the rickets work that concomitant with changes in bone structure there were changes in the composition of the blood (9). Studies made by Howland and Kramer indicated that on the high calcium-low phosphorus diets, calcium of the blood remains about normal, whereas the phosphorus content falls from the normal level to a 2 to 3 mg. level. The work of other investigators confirmed these findings, and more recently Holt (10) has reported studies of a quantitative nature relative to the equilibria concerned with the calcification of bone. According to their views, deposition of calcium salts in the bony matrix is influenced by the physicochemical equilibrium involved in the precipitation of $Ca_3(PO_4)_2$ from the blood serum. Normally, blood serum is greatly supersaturated with respect to calcium phosphate, and even in rickets the ion product $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$ exceeds the solubility product constant so that theoretically precipitation should take place. From their data, the ion product in healing or absence of rickets is greater than 8.0×10^{-25}, whereas in active rickets the product is below this figure (pH is assumed to be 7.35 in all cases). It would seem that $Ca_3(PO_4)_2$ should precipitate from such a serum, since the $K_{s.p.}$ for $Ca_3(PO_4)_2$ is 1×10^{-26}. But they have shown that this precipitation is a very

slow process except when the ion product greatly exceeds the solubility product constant,—so that in rickets the rate of precipitation is so slow that it is greatly exceeded by the rate of bone formation, and inadequately calcified tissue results. This reduced supersaturation of blood with $Ca_3(PO_4)_2$ is probably not the only factor which influences the calcification of rachitic bones, but at least it represents a rather definite relationship between blood composition on the one hand, and rachitic developments or healing on the other hand.

The fact that rickets could be produced experimentally and cured again by certain dietary measures, and the fact that the whole process could be followed by histological observations, gave promise of a procedure for testing the antirachitic potency of various samples of cod liver oil. Moreover, it seemed probable that rachitic developments and healing might be followed through reference to change in mineral content of the bone, and calcium and phosphorus content of the blood.

EXPERIMENTAL.

In harmony with the idea of correlating rachitic developments, and healing, with changes in blood and bone composition, the following plan of procedure was used.

Procedure.—Rats were fed on a rickets-producing diet for a certain preliminary period until rickets should develop, as determined by histological examination of the bones of a control group. Cod liver oil in minimal amounts was then added to the ration, and the rats were kept on this diet for a test period of a few days and then killed; the bones were examined histologically by the "line test" (11) to determine the degree of healing. A control group, however, was continued on the rickets-producing diet alone (*i.e.* no cod liver oil added) to the end of the test period, in order to indicate whether changes in the test group were really due to the influence of the cod liver oil. The femurs of typical rats from each group were analyzed for ash, calcium, and phosphorus; and serum from the pooled blood of each group was likewise analyzed for calcium and phosphorus content.

Preliminary Considerations.—Before launching upon the formulated plan, certain preliminary considerations were neces-

sary. The first of these was relative to the *choice of animals.* Since rickets is a disease of the growing individual, young growing rats were used, these being put upon the diet when between 50 and 60 gm. in weight, since animals of this size, though still growing rapidly, are capable of withstanding a rigorous dietary régime for some time. The animals were from the inbred stock colony, and were, therefore, very closely related, so that under a given treatment they were likely to respond in a uniform fashion.

The matter of *ultra-violet light influence* in retarding rachitic developments or facilitating healing was ruled out, in that the animals were kept in a north room darkened by keeping the shades drawn.

The *choice of a rickets-producing diet,* the determination of the *cod liver oil dosage,* and *length of test period,* were the other points to be settled upon before proceeding to a comparison of the curative effects of various samples of cod liver oil. And, with respect to these matters data accumulated in the course of experimental work.

Various diets had been proposed from time to time for use in producing experimental rickets, and among these were the Sherman and Pappenheimer Diet 84 (12), Steenbock's Ration 2965 (13), and various diets planned by McCollum and coworkers, particularly Diets 4338, 3143, and 4025, modified by substitution of Salt Mixtures 20 or 51. The composition of these diets is given below.

Sherman and Pappenheimer Diet 84.

Patent flour	95.00
Ca lactate	2.90
NaCl	2.00
Ferric citrate	0.10

Steenbock Yellow Corn Ration 2965.

Maize	76.00
Wheat gluten	20.00
$CaCO_3$	3.00
NaCl	1.00

McCollum Diet 4338.

Wheat	25.00
Maize	46.00
Wheat gluten	18.00
Gelatin	7.00
NaCl	1.00
$CaCO_3$	3.00

McCollum Diet 3143.

Wheat	33.0
Maize	33.0
Gelatin	15.0
Wheat gluten	15.0
NaCl	1.0
$CaCO_3$	3.0

McCollum Diet 4025.

(Modified Salt Mixture 20.)

Wheat germ	5.00
Salt Mixture 20	5.80
$CaCO_3$	1.50
Gelatin	10.00
Egg albumin	10.00
Wheat gluten	12.00
Agar agar	2.00
Dextrin	51.70
Butter fat	2.00

McCollum Diet 4025.

(Modified Salt Mixture 51.)

Wheat germ	5.0
Salt Mixture 51	6.1
$CaCO_3$	1.5
Gelatin	10.0
Egg albumin	10.0
Wheat gluten	12.0
Agar agar	2.0
Dextrin	51.4
Butter fat	2.0

Salt Mixture 51.

$CaCO_3$	1.5
KCl	1.0
NaCl	0.5
$NaHCO_3$	0.7
Ferric citrate	0.5
KH_2PO_4	1.7
MgO	0.2

Salt Mixture 20.

$CaCO_3$	1.5
KCl	1.0
NaCl	0.5
$NaHCO_3$	0.7
MgO	0.2
$FeSO_4$	0.2

The significant data obtained in feeding these rations to various lots of test animals (five rats in each lot) are tabulated in Table I.

The findings indicate that in satisfying growth requirements, any of the diets other than the Sherman-Pappenheimer Diet 84 would suffice. This diet is particularly severe, and though the animals develop rachitic bones, the changes are so extreme that it is doubtful whether there would be a sensitive response to vitamin D influence. Rats fed on Diet 4025 (modified) did not develop rickets, as evidenced either by the bone picture or blood analysis. These ion product values $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$ were calculated by the method of Holt (10), and values less than 8×10^{-25} indicate active rickets, whereas values greater than 8×10^{-25} indicate healing or absence of rickets. The other diets—Steenbock's Ration 2965 and McCollum's Diets 4338 and 3143—are satisfactory in causing the experimental animals to develop rickets, the bone picture and the blood findings confirming this view. Moreover, with cod liver oil added to these diets, a quick response was made in the healing of the rachitic bones, and in the adjustment of blood Ca:P values toward normal.

Of the three satisfactory diets, Diet 3143 was arbitrarily chosen for use throughout the remaining investigations. A few trials, wherein animals were placed upon the diet for varying periods

TABLE I.

Lot No.	Diet.	Days on experimental diet.	Average gain in weight.	Line test.		Blood.		
				Results.	Comment.	Ca per 100 cc. serum.	P per 100 cc. serum.	Ion product, $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$.
			gm.			mg.	mg.	
92	Sherman and Pappenheimer Diet 84.	22	0, some loss.	—	Not satisfactory as negative control.	11.52	2.70	6.22×10^{-25}
96	Steenbock Ration 2965.	21	30	—	Satisfactory negative control.	11.28	2.83	6.76×10^{-25}
95	" " 2965.	29	37	—	Good negative control.	10.56	1.43	1.45×10^{-25}
97	" " 2965 + cod liver oil from 21st to 29th day.	21–29	42	4+	Healing.	12.72	3.14	1.29×10^{-24}
94	McCollum Diet 4338.	22	27	—	Good negative control.	10.80	2.71	5.62×10^{-25}
101	" " 4338.	29	25	—	" "	10.32	1.30	1.21×10^{-25}
102	" " 4338 + cod liver oil from 21st to 29th day.	21–29	28	4+	Healing.	12.48	3.38	1.01×10^{-24}
103	McCollum modified Diet 4025 (Salt Mixture 20).	21	47		Bone well calcified; not typical rickets.	10.56	6.31	2.75×10^{-24}
99	McCollum modified Diet 4025 (Salt Mixture 51).	21	51		" "	10.80	6.98	3.89×10^{-24}
98	" " " " " " "	29	48		" "	10.32	4.28	1.38×10^{-24}
51	McCollum Diet 3143.	21	20	—	Good negative control.	10.76	3.11	6.92×10^{-25}
49	" " 3143.	30	28	—	" "	10.76	3.28	6.92×10^{-25}
57	" " 3143 + cod liver oil from 22nd to 30th day.	22–30	28	4+	Healing.	11.47	4.95	5.37×10^{-24}

before being killed for examination of sectioned bones, indicated that upon this dietary régime a grade of rickets satisfactory for line test technique would develop in 18 to 21 days, the 21 day period particularly giving rise to constant developments. With this ration, the 21 day period being chosen for development of the rachitic condition, experiments were carried out to determine satisfactory dosage of cod liver oil and appropriate length of test period. The nature and results of these experiments are tabulated in Table II.

These results seem to indicate that both the quantity of cod liver oil fed, and the length of time given for it to exert its curative effect, are factors in determining the degree of healing. Sample 255 A fed at a 0.25 per cent level for 8 days brought about a somewhat more pronounced healing than when fed for only 4 days. At a 0.5 per cent level this relationship was hardly pronounced, this amount of cod liver oil being too much to constitute a very critical level. With Sample 255 A, pronounced healing was obtained at a 0.125 per cent level on an 8 day test period. To lessen the test period to 4 days on a general procedure would scarcely be advisable since another sample of oil, No. 255 F, at a 0.125 per cent level for 8 days just failed evidently to bring about improvement of the rachitic condition (ion product less than 8.0×10^{-25}), though this failure was not evident at a 0.25 per cent level over an 8 day period. Note should be made here that neither the degree of healing indicated by the line test, nor the change in ion product values toward normal, is proportional to the cod liver oil intake. To illustrate the point particularly, the following figures may be abstracted from Table II for comparison:

Sample No.	Cod liver oil.	Length of test period.	Cod liver oil intake per rat.	Line test.	Ion product.
	per cent	*days*	*gm.*		
255 A	0.25	8	0.102	3+, 4+	1.05×10^{-24}
255 A	0.125	8	0.05	3+, 4+	2.63×10^{-24}
255 A	0.125	8	0.056	3+, 4+	2.14×10^{-24}

Evidently minimal quantities of the antirachitic factor will facilitate healing and cure of the rachitic condition, providing time

TABLE II.

Cod liver oil sample No.	Cod liver oil.	Length of test period.	Average food consumption per rat over test period.	Actual cod liver oil intake per rat.	Lot No.	Treatment diet.	Days from beginning of experiment.	Line test results.	Blood. Ca	Blood. P	Blood. Ion product, $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$.
	per cent	days	gm.	gm.					mg. per 100 cc.	mg. per 100 cc.	
255 A	0.5	4			51	Control to beginning of test period, Diet 3143.	21	−	10.76	3.11	6.92×10^{-25}
			20	0	50	Control to end of test period, Diet 3143.	25	(+), −, −	10.30	2.66	3.80×10^{-25}
			19	0.095	53	Test period, Diet 3143 + cod liver oil.	21–25	2+, 3+, 4+	11.47	3.89	1.23×10^{-24}
255 A	0.5	8			51	Control to beginning of test period, Diet 3143.	21	−	10.76	3.11	6.92×10^{-25}
			51	0	49	Control to end of test period, Diet 3143.	29	(+), −, −, −	10.76	3.28	6.92×10^{-25}
			40	0.20	52	Test period, Diet 3143 + cod liver oil.	21–29	3+, 4+	11.93	3.32	9.12×10^{-25}
255 A	0.25	4			51	Control to beginning of test period, Diet 3143.	21	−	10.76	3.11	6.92×10^{-25}
			20	0	50	Control to end of test period, Diet 3143.	25	+, −, −	10.30	2.66	3.80×10^{-25}
			20	0.050	55	Test period, Diet 3143 + cod liver oil.	21–25	+, 2+	10.99	3.80	1.07×10^{-24}
			23	0.058	56		21–25	+, 2+, 3+	12.17	3.63	1.26×10^{-24}

255 A	0.25	8			51	Control to beginning of test period, Diet 3143.	21	–	10.76	3.11	6.92×10^{-25}
			51	0	49	Control to end of test period, Diet 3143.	29	–	10.76	3.28	6.92×10^{-25}
			41	0.102	54	Test period, Diet 3143 + cod liver oil.	21–29	+, 2+, 3+	12.17	5.06	1.05×10^{-24}
255 A	0.125	8			51	Control to beginning of test period, Diet 3143.	21	–	10.76	3.11	6.92×10^{-25}
			51	0	49	Control to end of test period, Diet 3143.	29	–	10.76	3.28	6.92×10^{-25}
			46	0.056	57	Test period, Diet 3143 + cod liver oil.	21–29	2+, 3+	11.47	4.95	2.14×10^{-24}
			47	0.059	58		21–29	2+, 3+	11.70	5.55	2.63×10^{-24}
255 F	0.25	8			38 A	Control to beginning of test period, Diet 3143.	21	–	11.04	2.44	4.77×10^{-25}
			63	0	38 B	Control to end of test period, Diet 3143.	30	–	9.92	2.83	4.37×10^{-25}
			55	0.137	36	Test period, Diet 3143 + cod liver oil.	22–30	2+, 3+, 4+	11.37	3.70	1.23×10^{-24}
255 F	0.125	8			79	Control to beginning of test period, Diet 3143.	21	–	9.60	2.01	2.19×10^{-25}
			59	0	78	Control to end of test period, Diet 3143.	29	–	12.00	2.03	4.37×10^{-25}
			60	0.075	75	Test period, Diet 3143 + cod liver oil.	21–29	+, 2+, 3+	12.00	1.84	6.61×10^{-25}

Values greater than 8.0×10^{-25} indicate healing; values less than 8.0×10^{-25} indicate rachitic condition.

TABLE III.

Cod liver oil sample No.	Cod liver oil.	Length of test period.	Average food consumption per rat over test period.	Actual cod liver oil intake per rat.	Lot No.	Treatment diet.	Days from beginning of experiment.	Line test results.	Blood. Ca	Blood. P	Blood. Ion product, $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$.
	per cent	*days*	*gm.*	*gm.*					*mg. per 100 cc.*	*mg. per 100 cc.*	
255 A	0.125	8			51	Control to beginning of test period, Diet 3143.	21	–	10.76	3.11	6.92×10^{-25}
			51	0	49	Control to end of test period, Diet 3143.	29	–	10.76	3.28	6.92×10^{-25}
			45	0.056	57	Test period, Diet 3143 + cod liver oil.	21–29	2+, 4+	11.47	4.95	2.14×10^{-24}
			47	0.058	58		21–29	3+, 4+	11.70	5.55	2.63×10^{-24}
255 B	0.125	8			59	Control to beginning of test period, Diet 3143.	22	–	9.84	3.40	6.31×10^{-25}
			55	0	62	Control to end of test period, Diet 3143.	30	–	11.52		
			65	0.081	60	Test period, Diet 3143+ cod liver oil.	22–30	2+, 3+, 4+	12.58		
255 C	0.125	8			59	Control to beginning of test period, Diet 3143.	22	–	9.84	3.40	6.31×10^{-25}
			55	0	62	Control to end of test period, Diet 3143.	30	–	11.52		
			60	0.075	61	Test period, Diet 3143 + cod liver oil.	22–30	+, 2+, 3+, 4+	12.48		

255 C	0.25	8			29	Control to beginning of test period, Diet 3143.	21	–	10.16	4.5	1.21×10^{-24}
			52	0	31	Control to end of test period, Diet 3143.	30	–	9.92	2.5	3.31×10^{-25}
			59	0.147	30	Test period, Diet 3143 + cod liver oil.	22–30	+, –	11.13	3.44	1.02×10^{-24}
255 D	0.125	8			63	Control to beginning of test period, Diet 3143.	21	–	10.56	1.54	1.62×10^{-25}
			60	0	66	Control to end of test period, Diet 3143.	29	–	10.80	2.69	5.37×10^{-25}
			60	0.075	65	Test period, Diet 3143 + cod liver oil.	21–29	3+, 4+	11.28	3.63	1.03×10^{-24}
255 E	0.125	8			63	Control to beginning of test period, Diet 3143.	21	–	10.56	1.54	1.62×10^{-25}
			60	0	66	Control to end of test period, Diet 3143.	29	–	10.80	2.69	5.37×10^{-25}
			58	0.073	64	Test period, Diet 3143 + cod liver oil.	21–29	–, +, 2+, ?	12.48	4.44	2.24×10^{-24}
255 F	0.125	8			79	Control to beginning of test period, Diet 3143.	21	–	9.60	2.01	2.19×10^{-25}
			59	0	78	Control to end of test period, Diet 3143.	29	–	12.00	2.03	4.37×10^{-25}
			60	0.075	75	Test period, Diet 3143 + cod liver oil.	21–29	4+, –, +, 2+	12.00	1.84	3.80×10^{-25}

TABLE III—*Continued.*

Cod liver oil sample No.	Cod liver oil.	Length of test period.	Average food consumption per rat over test period.	Actual cod liver oil intake per rat.	Lot No.	Treatment diet.	Days from beginning of experiment.	Line test results.	Blood. Ca	Blood. P	Blood. Ion product, $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$.
	per cent	*days*	*gm.*	*gm.*					*mg. per 100 cc.*	*mg. per 100 cc.*	
255 G	0.125	8			79	Control to beginning of test period, Diet 3143.	21	–	9.60	2.01	2.19×10^{-25}
			59		78	Control to end of test period, Diet 3143.	29	–	12.00	2.03	4.37×10^{-25}
			65	0.081	76	Test period, Diet 3143 + cod liver oil.	21–29	+, –	12.24	2.50	6.61×10^{-25}
255 G	0.125	8			85 A	Control to beginning of test period, Diet 3143.	21	–	11.52	4.93	1.93×10^{-24}
			63	0	85 B	Control to end of test period, Diet 3143.	29	–	11.04	2.15	3.55×10^{-25}
			49	0.061	86	Test period, Diet 3143+ cod liver oil.	21–29	+ ?, 2+, 4+	10.56	2.62	4.57×10^{-25}
255 H	0.0125	8			79	Control to beginning of test period, Diet 3143.	21	–	9.60	2.01	2.19×10^{-25}
			59	0	78	Control to end of test period, Diet 3143.	29	–	12.00	2.03	4.37×10^{-25}
			43	0.054	77	Test period, Diet 3143 + cod liver oil.	21–29	4+, 2+, –	12.48	3.03	1.08×10^{-24}

is given for the process to materialize; and in any event, individual variations in the test animals will be contributing factors in the changes produced under the influence of cod liver oil.

On the basis of the above data and considerations, it was deemed advisable to administer the cod liver oils to be tested over a test period of 8 days and in dosages constituting 0.125 per cent of the ration. In this length of time, antirachitic vitamin, even though present in minimal amounts, would exert positive line test results, whereas less potent oils would give questionable or negative results.

After the details relative to choice of animals, preference of diet, preliminary control period, cod liver oil dosage, and length of test period had been decided upon, a series of experiments was undertaken involving the use in turn of seventeen different samples of oil. The findings are set forth in tabular form in Table III, though for the sake of brevity data are presented with respect to only eight of the seventeen samples. Just a word may be said here with regard to the technique involved in the operations indicated in this tabulation.

Technique of Line Test Procedure.—Since evidence of rickets develops at a different rate in the various bones, first in the ribs, next in the femur and tibia, and later in other bones, it was decided to section the tibia for microscopic examination, this being a convenient member to deal with, and one showing early developments of rachitic condition. Moreover, by the time changes were in evidence in the tibia, changes would have set in in the femur too, so that it would be safe to interpret chemical analysis of that bone as indicative of the condition in a rachitic bone. The tibiæ were dissected from the tissue (after the rats had been anesthetized with chloroform and killed by bleeding from the carotid artery which was laid bare) and a median longitudinal section made with a sharp scalpel, the two halves of each bone being immersed immediately in 1.0 per cent silver nitrate solution, the clean cut surfaces being kept uppermost. Light from a carbon arc lamp was focused for 1 to 2 minutes upon these immersed surfaces, and under this light examination of the bones made under a binocular microscope. Calcium deposits in the bone showed up distinctly black, bony trabeculæ and calcification in the provisional zone of calcification being therefore distinct. The objective of highest power being used, a fairly detailed histological picture was obtained, it being

possible to see in particular the arrangement of cells in the proliferative cartilage, and the calcium deposition (where present) between the cells of this zone. Bones giving a picture of active rickets are recorded as negative with regard to the line test. Such bones showed in the epiphyseal region, the cartilage band, with proliferation of cartilage cells toward the diaphyseal region; this proliferative zone dipped down in jagged strands due to invasion by sprouts of blood vessels. Immediately adjacent to this zone was a region free of calcification, a distinct metaphysis, lying, therefore, between the diaphyseal and epiphyseal region. Such was the nature of the bone picture in the great majority of the control cases, where rats had been kept on Diet 3143 for the 21 to 29 day periods. When cod liver oil was added to the ration, healing of the rachitic lesion began; that is, calcium salts began to deposit, and the normal structure of the bone began to be assumed again. Normally, healing may commence by deposition of calcium salts (chiefly phosphates) between the cells of the provisional zone of calcification. The zone of proliferative cartilage cells becomes narrower and of straighter, smoother margin and gradually the metaphysis becomes calcified. Changes such as those just described were seen to have taken place in the bones of those animals having had the cod liver oil incorporated in the ration after 21 days of Diet 3143. This deposition of salts in the provisional zone of calcification gave rise to a narrow black line (as seen under the microscope), the line being broken at times (since deposition started at several points at once), and at other times continuous, but of various widths in different cases, depending upon the degree of calcification. By use of the microscope an accurate idea of the progress of the calcification process could be obtained, and the degree of healing could be expressed as −, +, 2+, 3+, and 4+. Such is the significance of the results recorded in the tables, under the heading "Line test." With the naked eye, the black line of calcification (black, of course, due to the darkening in the light of the silver salt formed when the calcified bone was immersed in silver nitrate) could be discerned, but no accurate idea of its width or continuity could be had. Since the nicety of this line test for determining the antirachitic potency of a given cod liver oil depends upon detecting *early* changes toward healing, it is necessary to follow these changes quite accurately

under the microscope. Noting the rate of "filling in" by calcification of the metaphyseal region is not so well suited for judging the healing effect of a sample of oil, since ordinarily this process becomes pronounced after the deposition in the provisional zone of calcification; *i.e.*, healing in the metaphyseal region is a *later* stage in the process. On the other hand, note was made of some cases wherein the metaphysis did become peppered with calcium salt deposits before calcification in the provisional zone had begun. Microscopic examination is needed to detect which of the two types of healing progresses first. With the diet used in this series of experiments, *i.e.* Diet 3143, and with Steenbock's Ration 2965, and McCollum's Diet 4338, the former type of healing (*i.e.*, beginning with calcification in the provisional zone) was noted in all but a very few of the cases.

Technique of Analytical Methods.—Referring still to the operations noted in Table III, a word should be said about the blood analyses. Blood was obtained by clipping the exposed carotid artery of an anesthetized animal, the blood being collected directly into a centrifuge tube, and the blood from all the rats of a given group being collected in the same tube. The pooled sample was allowed to clot in the refrigerator, and centrifuged within 45 minutes or an hour. The serum, having been promptly separated from the clot, did not contain organic-bound phosphorus from the cells. The calcium in the serum was determined according to the method of Kramer and Tisdall (14), the permanganate for the titration being made up and standardized by the method of Halverson and Bergeim (15). The inorganic phosphorus of the serum was determined after the method devised by Tisdall (16).

Relationship of Ca:P Composition of Blood to Line Test Results.—From a study of the findings presented in (Table III) it is seen that the blood calcium falls but little under the influence of the rickets-producing diet. The value stays near the normal level, 10 to 11 mg. The drop in blood phosphorus from the normal level (8.5 mg.) is pronounced, though the actual value reached is a matter of individual variation in the test animals and not a matter determined by duration of the deficient dietary régime. Thus, after continuation of Diet 3143 for 21 days, blood analysis indicated in various groups such values for phosphorus as 4.5 mg. per 100 cc., 1.54 mg., 3.11 mg., 2.01 mg., *etc.;* or, upon continuing the diet to

TABLE IV.

Lot No.	Rat No.	Gain in weight in 8 day test period.	Food intake.	Cod liver oil intake.	Line test results.	Ion product, $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$.
		Sample 255 A.				
		gm.	*gm.*	*gm.*		
58	1	−2	47	0.059	4+	2.63×10^{-24}
	2	−1	47	0.059	3+	
	3	10	47	0.059	3+	
	4	4	47	0.059	3+	
	5	1	47	0.059	3+	
		Sample 255 D.				
65	1	9	68	0.085	4+, 3+	1.03×10^{-24}
	2	4	53	0.066	4+	
	3	6	59	0.074	3+	
	4	7	71	0.089	4+	
	5	4	50	0.062	3+	
		Sample 255 E.				
64	1	10	59	0.074	+	2.24×10^{-24}
	2	10	44	0.055	?	
	3	9	55	0.069	2+	
	4	13	68	0.085	−	
	5	14	64	0.080	+	
		Sample 355 F.				
75	1	9	77	0.096	4+	3.80×10^{-25}
	2	11	74	0.083	0, +	
	3	9	62	0.078	+	
	4	3	38	0.048	4+	
	5	5	51	0.064	2+	
		Sample 355 G.				
76	1	9	66	0.083	+	6.61×10^{-25}
	2	5	61	0.076	+	
	4	6	56	0.070	0	
	5	11	78	0.098	0	

TABLE IV—*Concluded.*

Lot No.	Rat No.	Gain in weight in 8 day test period.	Food intake.	Cod liver oil intake.	Line test results.	Ion product, $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$.
Sample 255 G.						
		gm.	*gm.*	*gm.*		
86	1	6	49	0.061	+	4.57×10^{-25}
	2	5	61	0.076	2+	
	3	6	49	0.061	2+	
	4	3	49	0.061	3+	
	5	−4	38	0.048	4+	
Sample 255 H.						
77	1	−13	35	0.044	4+	1.08×10^{-24}
	2	3	45	0.056	2+	
	3	−9	34	0.043	4+	
	4	−2	47	0.059	4+	
	5	5	55	0.069	−	

a 29 day period, a similar series of values was obtained—2.69 mg., 3.28 mg., *etc.*

In case of healing under cod liver oil influence, as indicated by positive line tests for example, there is to be noted an increase in the calcium and phosphorus content of the serum of the test group over that of the control groups. This increase, however, is not proportional to the degree of healing nor to the intake of cod liver oil. The significant point to note with regard to these blood findings is that the ion product falls below the value 8.0×10^{-25} in active rickets, only to increase beyond that value in healing or absence of rickets.

These data also indicate variations in the potency of the various samples of oil. More shall be made of this point later.

Mention was made previously of the fact that variations in the individual behavior of the test animals would constitute contributing factors in the changes produced under the influence of the antirachitic vitamin as contained in cod liver oil. This is well illustrated by the variable response of different rats undergoing similar treatment in a given group. The variations are set forth

in Table IV, in which are presented some of the data extracted from Table III, in combination with other findings.

These data do not lend themselves to an interpretation so accurate as to say that a given ion product, or a given increase in blood calcium and phosphorus of the test group over that of the control groups indicates a definite or exact degree of healing, and hence a definite potency of the curative oil. Neither can one say that a 4+ healing indicates that the oil producing it is 4 times as potent as one giving a + healing; for indeed, in any one group receiving any one sample of oil there may be a range in line test values from + to 4+. Nor is there any exact relationship between the degree of healing and the cod liver oil intake (even when consideration of only one sample is involved) so that certainly one cannot figure so many units of potency in so many gm. of oil. And, it is not correct to argue that line test variations are referable to variations in the growth of the animals, for in the first place such a relationship is not indicated by the data. In the second place, small increases in weight are not necessarily associated with bone growth, hence no conclusion can be drawn relative to the rate of deposition of calcium salts (*i.e.* healing) keeping pace with proliferation of osteoid tissue.

In other words, these data do not warrant the conclusion that a given sample of cod liver oil contains so many units of antirachitic vitamin, nor even that one oil is definitely so many times more potent than another oil. But, what may be said considering these findings in a general way, is that if the animals in the test group are growing fairly well and eating a normal amount of food, as compared with the control groups at least, then the healing of the rachitic condition (as indicated by positive line tests) must be due to the presence of antirachitic vitamin, since in the control groups, where the diet is unfortified, rachitic conditions persist. If a sufficient number of animals is used in testing out a given oil, the majority of animals will be found to respond in like degree. Moreover, if the ion product $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$ of the calcium and inorganic phosphorus of the blood is above the value 8.0×10^{-25}, healing of the rachitic condition is indicated, and positive line test results are thus confirmed. In like manner, negative line tests will be corroborated by an ion product value less than 8.0×10^{-25}. These two factors being considered, therefore, and

the responses of the several groups to various oils being compared, one sample may be determined as relatively more or less potent than another in respect to the antirachitic factor, vitamin D.

In addition to blood analyses and histological examination of the bones, a number of bone analyses was carried out in order to determine the nature of changes in bone composition occurring as rickets developed and again as healing progressed. It was hoped that results of chemical analysis could be correlated with histological changes.

Bone Analyses, Variable Results.—In order to determine just what changes in bone composition were due to cod liver oil influence, it was deemed wise to know first just what changes took place when the animal was kept on the rickets-producing diet without cod liver oil fortification. Upon this diet the ash content of the bone was found to fall far below the normal level, but, contrary to expectations, there was no graded decrease in ash content, as the experimental period upon this diet (No. 3143) was lengthened. The results obtained are indicated in the accompanying tabulation.

No. of days on Diet 3143.	Ash (average values.) per cent
21	30.15
22	25.53
25	29.20
28	28.10
29	27.98
30	26.93
32	23.43
33	31.98
36	27.20
37	27.74
40	22.11
41	28.37

These values are averages, four to twelve animals having been used in each group. The femurs of each animal were dissected out, cleaned, dried, crushed, and extracted in an extraction thimble with alcohol and ether until fat-free, the ash determinations for each rat being made on these dry fat-free bones. But in spite of the fact that the ash per cent was calculated on the basis of dry extracted bones, the averages are not very significant. It has

been shown by Hammett (17) and others that the composition of bones of the rat varies in accordance with the age and the sex of the animal. This influence could be eliminated by starting with males, only 50 to 60 gm., as test animals. In standardizing the procedure to this extent there is more nearly a graded falling off of

TABLE V.

Days on Diet 3143.	Males 50 to 60 gm. at beginning of experiment.		Ash in extracted bones.	Average ash.
	Rat No.	Lot No.		
			per cent	*per cent*
21	1	9	28.87	
	1	80	27.70	
	3	90	29.87	
	4	80	30.25	29.17
28	3	14	27.14	
	3	24	25.07	
	3	25	29.63	
	5	25	31.45	28.32
29	6	13	31.27	
	2	13	27.24	
	1	20	23.62	
	1	81	29.04	
	2	81	28.71	
	4	81	37.75	29.60
37	2	26	20.45	
	4	20	21.28	
	5	20	35.76	
	7	20	26.20	25.92
40	2	21	25.28	
	3	21	24.36	
	3	27	24.29	24.64

the ash per cent, as the number of days on the rickets-producing diet increases; the data in Table V illustrate this point. But even with this more standardized group of animals the individual variations are appreciable and it is questionable whether averages of such diverse values are of any significance. It would be absurd

TABLE VI.

Days from beginning of experiment.	Treatment.	Group.	Bone composition, per cent of extracted bone.* Ash.	Ca	P	Line test results (head of tibia).
21	Diet 3143.	Rats from Lots 18, 19, 20.	25.08	9.68	4.27	
29	" 3143.	Lot 20.	29.04	11.80	4.21	
21–29	Diet 3143 + 0.25 per cent cod liver oil, Sample 255 A.	Rat 7, Lot 17, right femur.	29.87	12.43	6.25	2+
		" 2, " 18, " "	33.14	10.37	5.82	2+
		" 2, " 18, left "	33.78	12.83	5.98	2+
		Group average.	31.82	11.87	6.01	2+
21	Diet 3143.	Lot 23.	23.60	7.62	4.34	–
29	" 3143.	" 21.	22.11	10.02	3.94	–
21–29	Diet 3143 + 0.25 per cent cod liver oil, Sample 255 B.	Rat 1, Lot 22, right femur.	26.45	11.57	4.13	2+
		" 2, " 22, " "	20.24	9.50	3.54	3+
		" 3, " 22, " "	23.95	10.60	3.69	2+
		Group average.	23.54	10.55	3.78	2+
21	Diet 3143.	Lot 29.	28.30	7.43	4.16	–
29	" 3143.	" 31.				–
21–29	Diet 3143 + 0.25 per cent cod liver oil, Sample 255 C.	Rat 1, Lot 30, right femur.	23.03	7.70	5.10	2+
		" 2, " 30, " "	30.61	11.30	5.68	+
		" 3, " 30, " "	27.16	9.53	5.07	–
		" 3, " 30, left "	28.00	10.07	5.07	+
		Group average.	27.20	9.65	5.23	+
21	Diet 3143.	Lot 32.	25.60	9.75	4.59	–
29	" 3143.	" 35.	25.17	8.92	4.49	–
21–29	Diet 3143 + 0.25 per cent cod liver oil, Sample 255 D.	Rat 3, Lot 33, both femurs.	25.54	8.56	4.61	2+
		" 4, " 33, right femur.	26.52	8.21	5.05	3+
		" 4, " 33, left "	26.46	7.86	4.98	3+
		Group average.	26.17	8.21	4.88	3+

TABLE VI—*Concluded.*

Days from beginning of experiment.	Treatment.	Group.	Bone composition, per cent of extracted bone.* Ash.	Ca.	P.	Line test results (head of tibia).
21	Diet 3143.	Lot 32.	25.60	9.75	4.59	–
29	" 3143.	" 35.	25.17	8.92	4.49	–
21–29	Diet 3143 + 0.25 per cent cod liver oil, Sample 255 E.	Rat 1, Lot 34, both femurs.	23.80	8.10	3.96	+
		" 2, " 34, " "	27.81	9.72	4.89	+
		" 3, " 34, " "	28.47	9.65	5.71	+
		Group average.	26.69	9.15	4.85	+
21	Diet 3143.	Lot 38 A.	34.43	11.67	6.44	–
29	" 3143.	" 38 B.	28.57	9.53	5.66	–
21–29	Diet 3143 + 0.25 per cent cod liver oil, Sample 255 F.	Rat 1, Lot 36, both femurs.	30.01	12.25	5.61	2+
		" 2, " 36, right femur.	42.35	14.58	7.23	+
		" 2, " 36, left "	40.16	16.03	7.67	2+
		" 4, " 36, right "	33.22	10.95	6.36	3+
		" 4, " 36, left "	32.77	11.77	6.03	4+
		Group average.	35.70	13.11	6.58	2+
21	Diet 3143.	Lot 38 a.	34.43	11.67	6.44	–
29	" 3143.	" 38 b.	28.57	9.53	5.66	–
21–29	Diet 3143 + 0.25 per cent cod liver oil, Sample 255 G.	Rat 2, Lot 37, both femurs.	28.53	10.24	5.78	3+
		" 3, " 37, " "	31.58	10.65	5.64	+
		" 4, " 37, " "	36.51	12.80	6.96	2+
		Group average.	32.20	11.23	6.12	2+
21	Diet 3143.	Lot 39.				–
29	" 3143.	" 42.	25.96	8.36	4.78	–
21–29	Diet 3143 + 0.25 per cent cod liver oil, Sample 255 H.	Rat 1, Lot 41, both femurs.	31.49	10.70	6.21	+
		" 3, " 41, " "	29.11	10.17	5.10	+
		Group average.	30.30	10.44	5.66	+

* Average values, except where given in detail.

to plot from these values a normal curve of reference whereby to judge the deviation produced under the influence of cod liver oil treatment of experimental animals. With no standard behavior on the basal diet, variations in ash content of the bones of animals subjected to vitamin D influence are of no special significance, especially since there is no decided trend of values over so brief a period as is needed for distinct line test results.

In Table VI are presented in some detail data with respect to the composition of bones of each test group, and bones of control groups to the beginning and the end of each test period. Granting that these average values might have some significance in spite of the diverse figures from which they are obtained, it is evident that

TABLE VII.

Percentage of ash (calculated on basis of dry extracted bone).			Line test results in test group.
Control to beginning of experiment (21 days).	Control to end of experiment (29 days).	Test group (*i.e.* cod liver oil treatment 21st to 29th day).	
23.60	22.11	23.54	2+
25.08	29.04	31.82	2+
28.30		27.20	+
25.60	35.17	26.17	3+
25.60	25.17	26.69	+
34.43	28.57	35.70	2+
34.43	28.57	32.20	2+
	29.11	30.30	+

about the most that can be said is that the ash per cent of the test group is generally, though not always, higher than that of the control groups. For the sake of comparison, the data relative to this relationship have been retabulated in Table VII. Moreover, it is seen that ash values of the test groups bear no particular relationship to the degree of healing (line test).

Analysis of the bones for calcium and phosphorus yielded values no more enlightening than those relative to the per cent of ash. In particular, individual animals varied greatly with reference to the calcium and phosphorus content of their bones, even when these animals had been subjected to identical treatment. (Refer to Table VI.) And, not only was there variation between

animals of the same group, but even in any given animal there was frequently a wide divergence between values obtained from the right and left femur respectively. Table VIII summarizes these divergencies. That the femora should not check with respect to their composition is not surprising in view of the fact that they did not always check relative to the deposit of salts along the pro-

TABLE VIII.

Rat No.	Lot No.	Femur.	Ca (dry extracted bone).	P (dry extracted bone).	Line test.
			per cent	*per cent*	
5	11	Right.	12.1	6.3	2+
		Left.	12.2	6.3	2+
2	18	Right.	10.37	5.82	2+
		Left.	12.83	5.98	2+
5	19	Right.	11.28	4.30	−
		Left.	11.20	4.47	−
7	20	Right.	12.29	6.95	−
		Left.	10.87	4.08	−
2	23	Right.	8.68	4.16	−
		Left.	6.38	3.95	−
3	25	Right.	11.70	5.55	4+
		Left.	10.78	5.42	4+
3	32	Right.	13.68	4.54	+
		Left.	10.20	4.96	+
1	35	Right.	9.69	4.13	−
		Left.	8.45	4.28	−
2	36	Right.	14.58	7.23	+
		Left.	16.03	7.67	2+
4	36	Right.	10.95	6.36	3+
		Left.	11.77	6.03	4+
1	40	Right.	8.38	4.94	2+
		Left.	8.15	4.60	3+
3	30	Right.	9.53	5.07	−
		Left.	10.07	5.07	+

visional zone of calcification. Moreover, as noted before, the line test does not always tell the whole story of calcification, since in the healing bones there is a greater or lesser deposit laid down in the metaphyseal region.

If those cases in which right and left femora failed to check were ruled out, and if it were granted that group averages for calcium and

TABLE IX.

Per cent of Ca (basis of dry extracted bone).			Per cent of P (basis of dry extracted bone).			Line test results in test group.
Control to beginning of experiment (21 days).	Control to end of experiment (29 days).	Test group (cod liver oil treatment 21st to 29th day).	Control to beginning of experiment (21 days).	Control to end of experiment (29 days).	Test group (cod liver oil treatment 21st to 29th day).	
9.68	11.80	11.87	4.27	4.21	6.01	2+
7.62	10.02	10.55	4.34	3.94	3.78	2+
7.43		9.65	4.16		5.23	+
9.75	8.92	8.21	4.59	4.49	4.88	3+
9.75	8.92	9.15	4.59	4.49	4.85	+
11.67	9.53	13.11	6.44	5.66	6.58	2+
11.67	9.53	11.23	6.44	5.66	6.12	2+
	8.36	10.44		4.78	5.66	+

TABLE X.

Cod liver oil sample No.	Percentage fed.	Line tests (majority of cases).	Ion product, $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$.	Potency.
255 A	0.125	3+, 4+	2.63×10^{-24}	Relatively high.
255 B	0.125	3+		" "
255 B	0.25	3+	7.94×10^{-24}	" "
255 C	0.25	+	1.02×10^{-24}	" low.
255 D	0.125	3+, 3+	1.03×10^{-24}	" high.
255 E	0.125	+	2.24×10^{-24}	Moderate.
255 F	0.125	2+, −	3.80×10^{-25}	Relatively low.
255 G	0.125	+, −	6.61×10^{-25}	" "
255 H	0.125	2+, −	1.51×10^{-24}	Moderate.
1	0.125	+, 2+	1.51×10^{-24}	"
2	0.125	4+		Relatively high.
3	0.125	+, 2+	8.32×10^{-25}	" low.
4	0.125	+, −	6.03×10^{-25}	" "
5	0.125	+, −	1.23×10^{-24}	" "
6	0.125	+	1.02×10^{-24}	Moderate.
7	0.125	3+	1.55×10^{-24}	Relatively high.
8	0.125	2+	1.55×10^{-24}	Moderate.
9	0.125	+	1.35×10^{-24}	"

phosphorus content of bones are significant, then a comparison might be made between these values for the test groups and those of the control groups. These values have been set forth for comparison in Table IX. Calcium and phosphorus values show no more of a decided trend than do the ash percentages. In general, though not always, the bones of the test group contain a higher percentage of calcium and phosphorus than do the bones of the control groups. No exact relationship exists, however, and in addition, no correlation with line test findings is evident.

It would seem from the above considerations that bone analyses lend no very definite information relative to the healing influence (*i.e.* antirachitic potency) of the oils under investigation. Not only is the information indefinite but in addition its reliability is to be questioned, since such diverse values are obtained even under identical experimental conditions, that averages are of questionable significance.

Comparison of Oils.—With judgment based upon the results of ion product values and the line test results, a comparison may be made relative to the potency of the various samples of cod liver oil assayed. The findings and conclusions are presented in Table X.

Approximately 500 rats have been used in the course of this investigation, and about 100 bone analyses have been made. The data are therefore sufficient to warrant rather definite conclusions.

DISCUSSION.

To consider data relative to bone composition as being of questionable significance in following rachitic developments is rather contrary to the general opinion. Thus, Dutcher (6) suggests that " . . . . chemical methods should be very useful in making accurate estimations, in a relatively short time, of the antirachitic potency of our common foods. It offers a quantitative method . . . . for we should be able to find, by feeding experiments, the minimum amount of any food which will prevent an appreciable fall of inorganic blood phosphorus or bone ash below the normal level of a normal 40 gm. rat or the minimum amount of any food which will bring about normal deposition of mineral matter in bones." Steenbock and coworkers make use of bone ash determinations as one set of criteria by which to judge the antirachitic effects of given rations (4, 13). Chick and coworkers

have also considered results of bone analyses reliable criteria in the matter of judging severity of rachitic condition (5).

Data obtained in the present investigation, however, indicate that there is scarcely a standard behavior of animals as evidenced by bone composition. There is a general trend of events, as indicated by ash values, for example,—in that the per cent of ash decreases as the severity of rickets increases. This decrease, however, is not by graded amounts. Moreover, as has been pointed out, individual variations are so pronounced that many animals of a given group show appreciable deviation from the average established for the group. This being the case, one would scarcely know whether to interpret comparatively slight deviation of test animals from the so called average as being due to cod liver oil influence, or as being a matter of individual variation again. In other words, the data here presented do not lend themselves for the establishment of a "normal" of behavior either in the case of animals on the rickets-producing diet alone, or animals subjected to cod liver oil treatment. That this is the case might be due to the basal diet (McCollum's Diet 3143) employed, for Steenbock has indicated (13) that data with regard to the ash of bones was much less uniform with Diet 3143 than when Steenbock's Ration 2965 was employed. Again, the method generally used (*i.e.*, cleaning, extracting, and ashing) to obtain the ash of bones may be the cause of the variations noted. Indeed, Kramer (7) has indicated that an erroneous impression is given, since by this method organic phosphorus is converted to phosphate.

Another point worthy of note is that the several investigators have employed various pathological changes as standards wherewith to follow rachitic developments and judge the significance of bone analyses. Gross lesions, radiographic evidence, and histological picture have been used—and late and early developments in the disease have thus been involved. Moreover, the observations have been made with respect to various bones—long bones in particular—but even in this group of bones rickets is first evidenced in the ribs, and later in the femurs and tibiæ.

In view of the factors just discussed it would be little wonder if the various investigators should arrive at different conclusions. Scrutiny of their data indicates for the most part that there is a downward trend of values for bone ash as the severity of rickets

increases. Moreover, where sufficient values are given the averages drawn are seen to be based in many cases upon rather diverse figures. The conclusions drawn as to the merits of bone analysis as an indication of rachitic developments, differ, seemingly, because of the interpretation given the findings and not because the data are actually at variance.

The trend of inorganic phosphate of the blood has been taken by some (4, 6) to indicate development of rickets, or progress of healing. The phosphate value was considered because it falls below the normal level in rickets, only to rise again as healing progresses, whereas the blood calcium remains at a fairly constant level regardless of the stage of the disease. This trend of events is indicated by the data presented in this paper, though the actual phosphate values are not to be correlated with any specific degrees of healing or of rickets. Moreover, since phosphate is deposited as the calcium salt in the calcified bone, it is more logical to consider the calcium: phosphorus relationship in predicting and discussing the possibilities of calcification of the rachitic bones. That the physicochemical relationship applying to the precipitation of this salt $Ca_3(PO_4)_2$ from solutions supersaturated with respect to it, holds in its deposition from blood serum, is quite well indicated by the work of Holt (10). It must not be considered, however, that mere precipitation explains the phenomenon of calcification of osteoid tissue and the deposition of calcium salts in the provisional zone of calcification. The findings of the present investigation confirm the conclusions of Holt that the ion product value is greater than 8.0×10^{-25} in healing or absence of rickets, and less than this value in active rickets.

In applying the procedure described for comparison of cod liver oils, a basal diet should be used which will satisfy the following requirements: (1) permit of fairly rapid growth of the test animals, (2) bring about a condition of florid rickets in 18 to 21 days, and (3) permit of healing of this condition within 8 days when cod liver oil fortification is added to the basal diet.

Should a sample of oil fail to evidence curative effects at the 0.125 per cent level, progressive increase of dosage would serve to establish a level at which healing would be produced. An idea of the relative potency of such an oil would thus be obtained.

SUMMARY.

1. The data obtained in this investigation do not bear out the anticipated possibility of determining the antirachitic potency of cod liver oils by following changes in bone composition.

2. Blood analyses calculated to give the ion product values $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$ indicate whether test animals show active rickets or healing.

3. Indications such as given by results of blood analyses can be correlated with line test (*i.e.* histological) observations in practically all cases. The line test picture as evidenced by the majority of a group of ten or fifteen animals should be indicative of the potency of a given oil.

4. Cod liver oils do vary with respect to their content of antirachitic vitamin.

CONCLUSIONS.

1. Application of the above method to assay of cod liver oil gives data which can be interpreted to indicate only relative potency of oils. Absolute comparison to the point of establishing so many arbitrary units of antirachitic factor per given weight of oil is not justified on the basis of data of the type obtained.

2. A given procedure is no more accurate than the least accurate of the factors involved; in particular, biological behavior, valuable as it is in indicating the general trend of results, is not subject to mathematical accuracy. It is the writers' opinion, therefore, that no biological assay with respect to the vitamin content of given substances will yield more than relative values.

3. The dosages ordinarily indicated, and the time period usually involved in the utilization of cod liver oil for prevention or cure of human rickets, admit of a sufficient factor of safety that it is probable that any of the better grades of oil upon the market afford protection.

BIBLIOGRAPHY.

1. United States Pharmacopœia X, Philadelphia, 1925, 496.
2. Poulson, E., *Lancet*, 1926, ccxi, 320.
3. McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., *J. Biol. Chem.*, 1921, xlvii, 507.
4. Bethke, R. M., Steenbock, H., and Nelson, M. T., *J. Biol. Chem.*, 1923–24, lviii, 71.

5. Chick, H., Korenchevski, U., and Roscoe, M. H., *Biochem. J.*, 1926, xx, 622.
6. Dutcher, R. A., Creighton, M., and Rothrock, H. A., *J. Biol. Chem.*, 1925, lxvi, 401.
7. Howland, J., Marriott, W. McK., and Kramer, B., *J. Biol. Chem.*, 1926, lxviii, 721.
8. Sherman, H. C., and Hessler, M. C., *J. Biol. Chem.*, 1927, lxxiii, 113.
9. Howland, J., and Kramer, B., *Am. J. Dis. Child.*, 1921, xxii, 105.
10. Holt, L. E., Jr., *J. Biol. Chem.*, 1925, lxiv, 579.
11. McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., *J. Biol. Chem.*, 1922, li, 41.
12. Sherman, H. C., and Pappenheimer, A. M., *J. Exp. Med.*, 1921, xxxiv, 189.
13. Steenbock, H., and Black, A., *J. Biol. Chem.*, 1925, lxiv, 263.
14. Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlvii, 475.
15. Halverson, J. O., and Bergeim, O., *J. Biol. Chem.*, 1917, xxxii, 159.
16. Tisdall, F. F., *J. Biol. Chem.*, 1922, l, 329.
17. Hammett, F. S., *J. Biol. Chem.*, 1925, lxiv, 685, 693.

ACETYL MONOSES.

IV. TWO ISOMERIC TRIACETYL METHYLLYXOSIDES.

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Recent work on the chemistry of sugars has furnished sufficient justification for the belief that a given monose in solution passes through a series of isomeric states. These isomeric forms differ from each other in their ring structures and are referred to as "lactal" isomers. In solution each lactal form is in equilibrium with the aldehydic form and the ratio of $\frac{\text{lactal form}}{\text{aldehydic form}}$ varies with the ring structure. Also the velocities of the two reactions, aldehydic form $\rightleftharpoons$ lactal form, are functions of the ring structure. There exists evidence to show that not only the ring structure but also the configuration of each sugar plays a part in determining what may be termed the stability of a given lactal structure. A most important contribution to this field of research was that of Fischer, Bergmann, and Rabe.[1] These authors prepared four isomeric triacetylmethylrhamnosides. Later, Dale[2] and Levene and Sobotka[3] described two isomeric forms of tetracetylmethyl- and ethylmannosides. On the basis of these observations Hudson[4] formulated very comprehensive conclusions regarding the ring structure of sugars. However, for a rigorous knowledge of the physical and chemical peculiarities of different lactal structures much more experimental material is needed than is now available.

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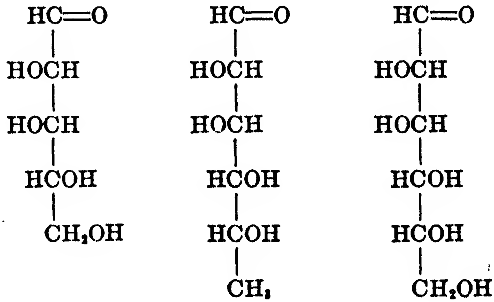
[1] Fischer, E., Bergmann, M., and Rabe, A., *Ber. chem. Ges.*, 1920, liii, 2362.

[2] Dale, J. K., *J. Am. Chem. Soc.*, 1924, xlvi, 1046.

[3] Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, 1926, lxvii, 759, 771.

[4] Hudson, C. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1424, 1434.

The two sugars from which the greatest number of lactal isomeric derivatives have been obtained are rhamnose and mannose. These two monoses are identical in the configuration of carbon atoms (2), (3), and (4). A third sugar with an identical



Lyxose. I. Rhamnose. II. Mannose. III.

configuration of the carbon atoms (2), (3), and (4) is lyxose. An attempt was therefore made to compare the conduct of this sugar when subjected to the same treatment which in the other two sugars led to the formation of the various ring isomers of the acetylated 1-methyl derivatives. The behavior of lyxose in some respects differed from that of the other two sugars and in others resembled them. In the case of *l*-rhamnose the substitution of the halogen in position (1) of the triacetylbromorhamnose led to three isomers. All of these may be regarded as β-glucosides inasmuch as they were dextrorotatory. One of the forms designated by Fischer, Bergmann, and Rabe[1] as the γ form differed from the other two in that one of the acetyl groups could not be removed by means of dilute alkalies. In the case of *d*-mannose two isomers were obtained under the same conditions, but apparently they belong to the β-glucosides inasmuch as both were levorotatory. One of the forms had the same peculiarities as the γ-rhamnose. In the case of *d*-lyxose, two isomers also were obtained of which one possessed the peculiarities of the γ-rhamnose and was levorotatory; the second form was identical with that obtained from α-methyllyxoside. The latter observation is rather unexpected inasmuch as in all past experience the acetylated halogen derivatives led to

β-glucosidic derivatives. The properties of the two isomeric forms of triacetylmethyl-*d*-lyxoside are given in Table I.

TABLE I.

Physical Properties of the Two Methyllyxoside Triacetates.

	M.p.	$[\alpha]_D^{25}$	
		Chloroform solution.	Methyl alcohol solution.
	°C.	*degrees*	*degrees*
α-Methyl-*d*-lyxoside triacetate.............	96	+30.4	+30.0
γ-Methyl-*d*-lyxoside "	90	−103.5	−98.3

EXPERIMENTAL.

Preparation and Measurement of α-Methyl-d-Lyxoside Triacetate.

α-Methyl-*d*-lyxoside was prepared according to the directions of Van Ekenstein and Blanksma[5] as modified by Phelps and Hudson.[6] The crystalline *d*-lyxose used showed mutarotation in the levo direction and the equilibrium value in water of $[\alpha]_D^{22} = -13.6°$. 13 gm. of once recrystallized α-methyllyxoside were dissolved in 26 cc. of dry pyridine by slight warming, cooled in ice, and 52 cc. of acetic anhydride added gradually. The flask was then stoppered and kept overnight at room temperature. The solution was poured into about 2 liters of ice water, 12 gm. of crystalline material being so obtained. The mother liquor was thrice extracted with chloroform and the extract washed twice with 2 per cent sulfuric acid, twice with sodium bicarbonate, and finally two or three times with water. The washed extract was dried with sodium sulfate and on evaporation yielded 10 gm. more of crystalline material.

On one recrystallization from ethyl alcohol the material showed the m.p. 95–96° and $[\alpha]_D^{25} = +30.3°$ (1.011 gm. substance, 25 cc. of chloroform solution, $\alpha = +2.45°$, 2 dm. tube) in chloroform solution. The once recrystallized material was allowed to stand

[5] Van Ekenstein, W. A., and Blanksma, J. J., *Z. Ver. deutsch. Zucker-Ind.*, 1908, lviii, 114.

[6] Phelps, F. P., and Hudson, C. S., *J. Am. Chem. Soc.*, 1926, xlviii, 503.

several days, with occasional shaking, under 70 per cent ethyl alcohol, filtered, and washed with absolute ethyl alcohol. The so purified material melted at 96° and yielded $[\alpha]_D^{22} = +30.2°$ (1.029 gm. substance, 25 cc. of chloroform solution, $\alpha = +4.98°$, 4 dm. tube). After three further recrystallizations from ethyl alcohol the substance melted at 96° and gave the following rotation value in chloroform solution:

$$[\alpha]_D^{24} = \frac{+\ 4.90° \times 100}{4 \times 4.00} = +30.6°.$$

The melting point of 96° and the rotation value of $[\alpha]_D^{25} = +30.4°$ in chloroform solution may be accepted for pure α-methyl-*d*-lyxoside triacetate. The substance crystallizes in prisms or prismatic needles and is very soluble in ether, chloroform, and methyl alcohol, less soluble in ethyl alcohol, slightly soluble in petroleum ether, and appreciably soluble in water at room temperature. It reduces Fehling's solution only after acid hydrolysis. The material analyzed as follows:

3.295 mg. substance: 5.990 mg. CO_2, 1.905 mg. H_2O.
0.1256 gm. " : 0.0984 gm. AgI.
100 mg. " : 10.55 cc. 0.1 N NaOH.
$CH_3O \cdot C_5H_6O_4 \cdot (CH_3CO)_3$.
Calculated. C 49.63, H 6.23, OCH_3 10.69, acetyl 10.3 cc. 0.1 N NaOH.
Found. " 49.67, " 6.46, " 10.34, " 10.55 " 0.1 " "

Preparation and Measurement of γ-Methyl-d-Lyxoside Triacetate.

10 gm. of crystalline α-*d*-lyxoside tetraacetate (m.p. 93–94°, $[\alpha]_D^{25} = +25°$ in chloroform solution) were treated with 50 cc. of cold (14°) glacial acetic acid nearly saturated in the cold with dry hydrogen bromide. The material dissolved rapidly and the solution was allowed to stand 20 minutes at room temperature. It was then poured into a separatory funnel holding 200 cc. of chloroform at a temperature slightly above the freezing point of chloroform and washed rapidly three times with ice water. The chloroform solution was then rapidly dried in the cold by shaking with several portions of dried sodium sulfate and allowed to stand overnight in the refrigerator. The sodium sulfate was removed by filtration and for this experiment one-half of the solution, thus corresponding to 5 gm. of the tetraacetate, was concentrated to a

thick syrup at 25° under reduced pressure. Repeated attempts to crystallize this syrup containing bromine failed.

5 cc. of very dry methyl alcohol were added to the syrupy bromo compound followed by 2 cc. of freshly distilled Kahlbaum quinoline. The solution was allowed to stand at room temperature for 90 minutes. 7 gm. of dry silver carbonate were then added and the mixture shaken vigorously until the first rapid effervescence was over, after which the mixture was shaken overnight in the cold on the shaking machine. The silver salts were then removed by filtration and washed with dry methyl alcohol. The extract was concentrated under reduced pressure at 20–25° to a thick broth of crystals. These were washed by decantation with several portions of petroleum ether to remove most of the quinoline. The residue, highly colored by silver compounds, was kept overnight in a desiccator in the cold. The syrup was removed from the crystals by treating the mixture with very cold ethyl alcohol and filtering rapidly. The material was recrystallized from methyl alcohol by cooling in a bath of solid CO_2 and ethyl alcohol, filtering rapidly, and washing with very cold methyl alcohol, 0.5 gm. of crystals being so obtained. By concentrating the filtrate from the first crystals to a syrup, taking this up in methyl alcohol, cooling and nucleating, 0.2 gm. more of crystals was obtained.

The procedure for preparing this compound was repeated with some modification. The time of standing in the brominating mixture was increased to 2 hours and the chloroform solution washed once with a cold solution of sodium bicarbonate and twice with ice water. The syrupy bromo compound from 7.5 gm. of lyxose tetraacetate was treated with a previously mixed solution of 15 cc. of dry methyl alcohol and 6 cc. of quinoline. It was allowed to stand for 90 minutes, as before, and then diluted with about 100 cc. of dry methyl alcohol, and a little solid phenolphthalein added. The acidity was neutralized by adding a dry methyl alcoholic solution of sodium methylate until the first faint pink color appeared, and then discharging this with carbon dioxide gas. The solution was concentrated at 25° under reduced pressure and the resultant syrup extracted several times with petroleum ether to remove quinoline. The neutralization with sodium methylate instead of silver carbonate had the advantage of rapidity and dispensed

with the troublesome reduction of silver salts to metallic silver on concentrating the solution. The extracted syrup was taken up in dry ether and filtered from sodium bromide and some reducing material, concentrated under reduced pressure at 25°, and the syrup again extracted with petroleum ether. The extracted material was then taken up in dry methyl alcohol, cooled in a bath of carbon dioxide and alcohol, and 0.5 gm. of crystals obtained on nucleation.

The substance was recrystallized from methyl alcohol by dissolving at room temperature, filtering, cooling in a bath of solid carbon dioxide and ethyl alcohol, filtering rapidly, and washing with very cold methyl alcohol. After one recrystallization, the substance melted sharply at 90° and showed $[\alpha]_D^{22} = -103.7°$ (0.1918 gm. substance, 5.01 cc. chloroform solution, $\alpha = -7.94°$, 2 dm. tube) in chloroform solution. After two more recrystallizations with 30 per cent loss the substance melted at 89.5° and showed the following rotation in chloroform solution:

$$[\alpha]_D^{22} = \frac{-8.25° \times 100}{2 \times 4.00} = -103.1°.$$

The melting point of 90° and the rotation value of $[\alpha]_D^{22} = -103.5°$ in chloroform solution may be accepted for pure γ-methyl-*d*-lyxoside triacetate. When slowly crystallized, the substance appears as nearly perfect hexagonal plates, but it crystallizes ordinarily as elongated hexagonal plates. The substance gives combustion and methoxyl values for a completely acetylated methyllyxoside but the acetyl value shows that one acetyl group is resistant to alkaline hydrolysis. The compound reduces Fehling's solution only after acid hydrolysis.

The analysis follows.

4.960 mg. substance: 9.000 mg. CO_2, 2.820 mg. H_2O.
0.1262 gm. " : 0.0986 gm. AgI.
100 mg. " : 7.2 cc. 0.1 N NaOH.

$CH_3O \cdot C_5H_6O_4 \cdot (CH_3CO)_3$.

Calculated. C 49.63, H 6.23, OCH_3 10.69, acetyl 10.3 cc. 0.1 N NaOH.
Found. " 49.48, " 6.36, " 10.31, " 7.2 " 0.1 " "

$C_6H_{12}O_6\ (CH_3CO)_2$.

Calculated. Acetyl 6.9 cc. 0.1 N NaOH.
Found. " 7.2 " 0.1 " "

Reaction Products of Syrupy Bromoacetyl Lyxose with Methyl Alcohol and Silver Carbonate.

The syrup obtained by shaking the syrupy bromoacetyl lyxose overnight in the cold with methyl alcohol and silver carbonate, representing four runs from a total of about 45 gm. of lyxose tetraacetate, was taken up in dry ether solution. After filtration from some amorphous material and removal of the ether under reduced pressure, the syrup was distilled. The first distillate, boiling at about 150° at 1.5 mm., 4 gm. in amount, was taken up in hot water and on cooling and standing deposited 0.6 gm. of crystals. These reduced Fehling's solution only after acid hydrolysis, melted at 90°, and gave a mixed m.p. with γ-methyllyxoside triacetate (m.p. 90°) of 65–75°.

Crystals of two forms of acetylated methyllyxosides having been obtained, a final experiment with the bromoacetyl compound was made, using these as nucleating material. 30 gm. of lyxose tetraacetate were converted into the bromo compound in two runs of 15 gm. each, according to the method previously described. The combined syrupy product was dissolved in 200 cc. of dry methyl alcohol, cooled to about 12°, and dry sodium sulfate and 40 gm. of silver carbonate added and shaken overnight at 12° on the shaking machine. The mixture was filtered, washed with dry methyl alcohol, concentrated to low volume, cooled in a bath of solid carbon dioxide and alcohol, and nucleated with the γ-methyllyxoside triacetate. 3.4 gm. of crystals were so obtained. After two recrystallizations from methyl alcohol 2.5 gm. of crystals were obtained which melted at 89° and gave a mixed melting point with crystals of the γ form (melting at 89.5°) of 88–89°. The rotation in chloroform solution was as follows:

$$[\alpha]_D^{25} = \frac{-8.16° \times 100}{2 \times 4.00} = -102°.$$

The substance was thus identified as the γ-methyl-*d*-lyxoside triacetate (m.p. 90°, $[\alpha]_D^{22} = -103.5°$).

The filtrate from the 3.4 gm. of the γ form was concentrated under reduced pressure to a syrup. This was taken up in hot water, cooled, and nucleated with the crystals of the second form obtained by distillation. After standing overnight in the cold,

the crystals were filtered off and washed with ice water. The aqueous filtrate was extracted twice with a small amount of ether, the etheral solution concentrated to a syrup, which yielded a further small amount of crystals from hot water.

The two crops of crystals (1.0 gm.) obtained from hot water were combined with the crystals from the distillation experiment (1.6 gm. total) and recrystallized once from methyl alcohol. The product (1.1 gm.) melted at 94° and gave a mixed melting point

TABLE II.

Acetylated Methyllyxosides.

Optical Changes in Methyl Alcohol Containing 0.1 Per Cent Hydrogen Chloride.

$l = 2$ dm. $\lambda = 5892$ Å.

γ form. c = 1.47, t = 22°.			α form. c = 2.95, t = 23°.		
Time.	α	$[\alpha]_D$	Time.	α	$[\alpha]_D$
min.	*degrees*	*degrees*	*min.*	*degrees*	*degrees*
0		−98.3	0		+30.0
1	−2.2	−75			
2	−1.5	−51			
3	−1.1	−37	3	+1.80	+30.5
5	−0.68	−23			
7.5	−0.58	−20			
10	−0.48	−16			
12.5	−0.50	−17			
15	−0.51	−17	15	+1.82	+30.9
			35	+1.74	+29.5
			hrs.		
			1	+1.72	+29.2
			2	+1.71	+29.0
			19	+1.39	+23.6

with crystals of pure α-methyllyxoside triacetate (m.p. 96°) of 94°. The rotation in chloroform solution was as follows:

$$[\alpha]_D^{25} = \frac{+2.51° \times 100}{2 \times 4.01} = +31°.$$

The substance was thus identified as α-methyl-*d*-lyxoside triacetate (m.p. 96°, $[\alpha]_D^{25} = +30.4°$).

Behavior of the Two Forms of Acetylated Methyllyxosides with Methyl Alcohol Containing Hydrogen Chloride.

The data in Table II show that the γ form of acetylated methyllyxoside gives a remarkably rapid optical rotation change in methyl alcohol containing approximately 0.1 per cent hydrogen chloride by weight. On the other hand, the α form shows only a very slight change.

The initial values given in Table II were determined in separate experiments in pure methyl alcohol. The solution of the γ form (0.1518 gm.) in methyl alcohol containing hydrogen chloride was neutralized with silver carbonate at the end of 20 minutes, filtered, and the filtrate concentrated under reduced pressure. 0.0707 gm. of syrup was obtained. This did not crystallize. An acetyl determination in aqueous methyl alcohol gave 0.54 cc. of 0.1 N NaOH consumed. This is 0.76 cc. on the basis of 100 mg., the calculated value for the triacetate being 10.3 cc. The substance was thus nearly completely deacetylated by the short treatment with dry methyl alcohol containing 0.1 per cent of hydrogen chloride.

STUDIES IN NUTRITION.

I. GROWTH, REPRODUCTION, AND LACTATION ON DIETS WITH DIFFERENT PROPORTIONS OF CEREALS AND VEGETABLES.

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With the Cooperation of Ruth E. Evans and Emily M. Bloomfield.

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(Received for publication, May 7, 1928.)

During the past 5 years an extensive study of children's dietaries has been in progress with a view to developing some simple practical method of judging nutritive value without recourse to detailed and laborious calculations. Some easily applied measuring device was especially needed for groups of children whose dietaries could not possibly be evaluated individually. No true criterion of the nutritive value of a diet is afforded by menus alone. As Dr. Ruth Wheeler has aptly remarked in the American Red Cross Textbook on Food and Nutrition, "On paper it would look adequate; the nutrients are all there. But that is the real trouble, the nutrients are in the menu but not in the family."

In a preliminary study of well selected diets of moderate cost for children ranging in age from 5 to 15 years, adequate in all respects according to modern standards, it was found that about 25 per cent of their total calories was in the form of cereal foods and from 12 to 18 per cent in the form of vegetables (1). In reducing the cost of any diet the natural course is to increase the amount of bread and other cereal foods because these are among the cheapest items in the dietary. A long and detailed study of the dietaries of children in several institutions was made to try to find out what proportion of cereals was most favorable to health and growth (2). Children were found to be in good health and making good growth on diets in which the proportion of cereal food varied from 23 to 52 per cent of the total calories and that of fruit and vegetables from 10 to 17 per cent. These investigations

covered many months, in some cases several years, but it was not found possible from the reports of medical examinations and weight and height records of the children to determine whether there was any particular advantage in the lower proportion of cereal and the higher one of vegetables.

Any such experiment on human beings can be continued through only a relatively small part of the life of the individual. 2½ years in the life of a child corresponds to a single month in the life of an albino rat, a period entirely too short for studying the influence of any but the most inadequate diets on growth, to say nothing of the effect on the species as shown by reproduction and lactation. It seemed necessary, therefore, to undertake a laboratory investigation which might be a more severe test, and perhaps reveal deficiencies which did not appear in the short trials possible with the children. At present we have no final criterion of the nutritive value of any diet except a biological one. Even if we subject the food to chemical analysis we cannot by this method study the vitamins, nor the effect which one dietary component may have upon the quantitative requirement for others. It has been shown, for instance, that rickets (3) may be induced by a disproportion between calcium and phosphorus and that magnesium is unfavorable to the utilization of calcium, especially when the ration is low in calcium and phosphorus (4, 5). Accordingly a series of experiments was begun with the albino rat with rations based on our findings in our studies of children. The cereal foods in the dietary were represented by white wheat flour as being the type of commonly used cereal food making the poorest contributions to the diet aside from total calories. If diets of which this constituted an important part proved adequate, it would seem safe to assume that others in which less highly refined cereals are included would also do so. Since the importance of milk as a special item in children's dietaries has been amply demonstrated (6, 7), this investigation has been limited to diets in which not less than 25 per cent of the total calories came from milk.

The results of the work with three diets will be reported in this paper. In Diet H white flour furnished 50 per cent of the total calories and assorted vegetables 10 per cent; in Diet M, the flour furnished 37.5 per cent and the same kinds of vegetables 15 per cent; and in Diet L, the flour furnished 25 per cent and the

vegetables 20 per cent. In each of the three diets whole milk powder furnished 25 per cent of the total calories. To represent the fruit-vegetable quota of the human diet vegetables were chosen which may be regarded as good staples in a moderately priced diet for growing children, the daily inclusion in the child's diet of a potato, a leafy vegetable, and a recognized source of vitamin C, and additional staple vegetables of low cost which are generally available being allowed for. Potatoes, spinach, tomatoes, carrots, onions, and dried peas constituted the vegetable

TABLE I.

Composition of Diets.

	Weights of dry ingredients per 100 gm.			Total calories from each food.		
	Diet H.	Diet M.	Diet L.	Diet H.	Diet M.	Diet L.
	gm.	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
Flour	56.65	43.23	29.30	50.00	37.50	25.00
Milk	19.70	20.04	20.38	25.00	25.00	25.00
Dextrin	5.00	9.67	13.46	5.00	9.50	12.00
Butter substitute*	4.26	5.43	6.61	8.00	10.00	12.00
Meat residue	2.16	3.30	5.59	2.00	3.00	5.00
Peas, dried	3.18	2.16	1.10	3.00	2.00	1.00
Potatoes	5.99	11.29	16.78	5.25	9.75	14.30
Carrots	1.34	2.52	3.77	1.40	2.60	3.80
Spinach	0.14	0.24	0.37	0.14	0.26	0.38
Tomato	0.48	0.90	1.33	0.11	0.20	0.29
Onions	0.12	0.22	0.31	0.11	0.20	0.29
Sodium chloride	1.00	1.00	1.00			

* Containing vitamin A equal to good butter.

assortment, all being fed to the experimental animals in the dried, powdered form. The legumes (peas) were limited to quantities not exceeding 3 per cent of the total calories, as it is not easy to make large amounts acceptable in human diets. A butter substitute containing vitamin A was used as experimental work in our laboratory with good butter and this substitute had already shown that there was no significant difference in the content of vitamin A. Dextrin was used as the equivalent of sugar, in quantities not exceeding what is reasonably safe in children's diets. Meat residue furnished enough additional protein to keep

the total protein calories at the desired level of 14 to 15 per cent of the total calories. The composition of the experimental rations is shown in Table I and their estimated nutritive values, calculated to the basis of 100 calories, are given in Table II.

Each diet had a protein content within 10 per cent of Sherman's Diet 13 (one-third whole milk powder, two-thirds whole wheat

TABLE II.

*Nutritive Value per 100 Calories.**

	Diet H.	Diet M.	Diet L.
	gm.	*gm.*	*gm.*
Protein	3.7	3.7	3.9
Fat	2.4	2.6	2.8
Carbohydrate	15.8	15.4	14.7
Calcium	0.050	0.051	0.054
Phosphorus	0.056	0.056	0.057
Iron	0.0004	0.0005	0.0006
Magnesium	0.011	0.012	0.013
Potassium	0.118	0.147	0.177
Sodium	0.033	0.035	0.037
Chlorine	0.053	0.054	0.055
Sulfur	0.042	0.038	0.034
	units	*units*	*units*
Vitamin A†	240–400	333–621	424–842
" B	22	22	23
" C (cooked food)	1	2	3

* Protein and ash constituents are calculated from average analyses except in the case of iron in the meat residue, which was kindly determined by Dr. E. J. Quinn, Instructor in Chemistry, Columbia University. The units of vitamins A and B are calculated on weight of fresh uncooked food materials from data in Sherman's "Chemistry of Food and Nutrition." The data on vitamin C are for cooked food and have been obtained by laboratory studies with guinea pigs and calculated to units as suggested by Sherman.

† Estimated on minimum and maximum number of units per pound.

and common salt equal to 2 per cent of the flour, with the addition three times weekly of lean beef and lettuce), on which fully twenty-five generations have been successfully reared, and about the same fat and carbohydrate distribution. Diet H had only half as much iron as is found in Diet 13, but the iron content of Diet L was within 10 per cent of that of Diet 13. The three

experimental diets had nearly equal proportions of calcium and phosphorus and the total quantity of each did not vary from the lowest to the highest by more than 10 per cent. The total amount of calcium in the three diets was about 70 per cent of that in Diet 13, and of phosphorus, about 50 per cent, the phosphorus being higher than the calcium in Diet 13.

The experimental diets were all higher in vitamin A than Diet 13, Diet L having according to our estimate about 4 times as much and Diet H at least twice as much.[1] Our diets differed little among themselves in content of vitamin B, but had only about half as much as Diet 13. Whether vitamin F (the antineuritic vitamin) and vitamin G (the pellagra-preventing vitamin) were as well proportioned as in Diet 13 it is not yet possible to say. Whole wheat has been shown by Sherman and Axtmayer (8) to be rich in vitamin F and milk to be rich in vitamin G. Goldberger and Wheeler (9) have reported tomatoes and carrots as containing the pellagra-preventive fraction, and it is not unlikely that some of our other vegetables supplement the milk in this way.

Selection and Care of Experimental Animals.

The experimental animals were all from stock bred and reared on Sherman's Diet 13. Four lots of rats were started on each diet. Two of these were composed of one male and two females each, the others of one male and one female each—ten animals in all. Animals in each lot were taken from their mothers at the age of 28 days and so matched that brothers and sisters were represented in the lots on each diet. In the second, third, and fourth generations, two lots of one male and two females each were continued on the experimental diet of their parents whenever possible.

In addition to the customary weekly weight and food records, careful breeding records were kept, so that the age of females at birth of first young, infant mortality, and weight of young at weaning time might be taken into consideration in judging the relative value of the diets. All animals were autopsied at death

[1] Dr. Florence MacLeod was unable to use young from our diets in tests for vitamin A because it took too long to deplete their bodies of the higher store as compared with the condition obtained with Diet 13. (Private communication.)

TABLE III.

Food Consumption in Typical Cases.

Calories per gm. of rat per day.

	Diet H.					Diet M.					Diet L.				
Rat No.	B94	B96	B228	B454	Average.	B95	B117	B229	B455	Average.	B101	B116	B230	B456	Average.
Age. wks.															
5–6	0.47	0.47	0.48	0.42	0.46	0.41	0.48	0.50	0.44	0.46	0.34	0.57	0.40	0.44	0.44
7–8	0.37	0.43	0.43	0.38	0.40	0.43	0.38	0.44	0.39	0.41	0.38	0.44	0.44	0.42	0.42
9–10	0.32	0.34	0.33	0.30	0.32	0.32	0.35	0.32	0.31	0.32	0.36	0.35	0.33	0.35	0.35
11–12	0.30	0.31	0.27	0.29	0.29	0.28	0.29	0.27	0.29	0.28	0.33	0.30	0.30	0.29	0.31
13–14	0.29	0.26	0.23	0.26	0.26	0.28	0.25	0.25	0.25	0.26	0.29	0.26	0.26	0.22	0.26
15–16	0.24	0.25	0.22	0.21	0.23	0.25	0.24	0.23	0.23	0.24	0.25	0.24	0.22	0.20	0.23
17–18	0.22	0.22	0.20	0.17	0.20	0.22	0.21	0.20	0.20	0.21	0.21	0.20	0.21	0.18	0.20
19–20	0.21	0.22	0.20	0.20	0.21	0.19	0.22	0.17	0.20	0.20	0.21	0.19	0.19	0.16	0.19
21–22	0.20	0.19	0.20	0.22	0.20	0.19	0.17	0.19	0.21	0.19	0.18	0.19	0.20	0.19	0.19
23–24	0.18	0.19	0.16	0.23	0.19	0.19	0.18	0.19	0.22	0.20	0.17	0.19	0.19	0.20	0.19
25–26	0.19	0.18	0.15	0.18	0.18	0.17	0.16	0.18	0.19	0.18	0.17	0.17	0.16	0.17	0.17
27–30	0.18	0.16	0.16	0.20	0.18	0.16	0.16	0.14	0.19	0.16	0.15	0.16	0.17	0.18	0.17
31–34	0.15	0.15	0.16	0.17	0.16	0.15	0.16	0.15	0.16	0.16	0.16	0.16	0.15	0.15	0.16
35–38	0.17	0.14	0.18	0.16	0.16	0.15	0.15	0.17	0.16	0.16	0.15	0.15	0.20	0.17	0.17
39–42	0.14	0.13	0.16	0.15	0.15	0.14	0.13	0.19	0.15	0.15	0.14	0.13	0.19	0.15	0.15
43–46	0.17	0.15	0.13	0.16	0.15	0.13	0.13	0.18	0.16	0.15	0.13	0.13	0.15	0.16	0.14
47–50	0.16	0.15	0.16	0.15	0.16	0.15	0.15	0.18	0.17	0.16	0.16	0.15	0.16	0.15	0.16
51–52	0.17	0.14	0.14	0.15	0.15	0.15	0.14	0.18	0.19	0.17	0.15	0.14	0.16	0.14	0.15

and examined for signs of infection, rickets, or other disease. The general technique has been that described by Sherman and Campbell (10).

In order to set a practical limit to the size of the colony, the animals were regularly killed and autopsied at the age of 12 months, it being thought that comparison of the breeding records would be fair, since females have most of their young before the close of their 1st year. All the young were kept with their mothers until 28 days of age, when those not needed to carry on the experiment were killed. On each diet the number of animals reared to 1 year of age was approximately 40. In making up the lots for each succeeding generation, healthy animals of medium size were consistently selected. On every diet a fifth generation has been produced, but as there is little indication that conclusions would be changed in any way by including it, and as the records for it are still incomplete, this paper is chiefly based on the findings through three generations.

TABLE IV.

Weight Gains from 28th to 56th Day of Life.

Diet.	No. of males.	No. of females.	Range of gain.		Average of gain.	
			Males.	Females.	Males.	Females.
			gm.	*gm.*	*gm.*	*gm.*
H	22	32	48–96	39–73	69 ± 1.8	55 ± 1.0
M	20	33	42–94	26–80	75 ± 2.0	55 ± 1.2
L	24	36	35–111	31–76	80 ± 2.1	55 ± 1.2

Food Consumption.

The food consumption on all three diets was normal in every way. The average daily food intake calculated to calories per gm. of rat is given in Table III along with some typical cases on each diet. The gain in weight for each 1000 calories consumed was calculated for the diet of median value (Diet M) and found to be 70 gm., as compared with the average for Diet 13 of 77 gm.

Growth.

The gains in weight for the 1st month on the experimental diet were, for the females, the same on all diets, but the males

made slightly better growth as the proportion of flour in the diet was reduced. Weight gains for both sexes from the 28th to the 56th day are shown in Table IV.

The growth of animals on all three diets was excellent as is indicated by comparison with the Donaldson curve. Starting with good average weight, they more and more exceeded the Donaldson average as they grew older. Each successive generation grew at a little better rate than the preceding one. This was more marked on Diets M and L than on Diet H. Growth curves for each generation of males up to the 228th day are shown in Chart I.

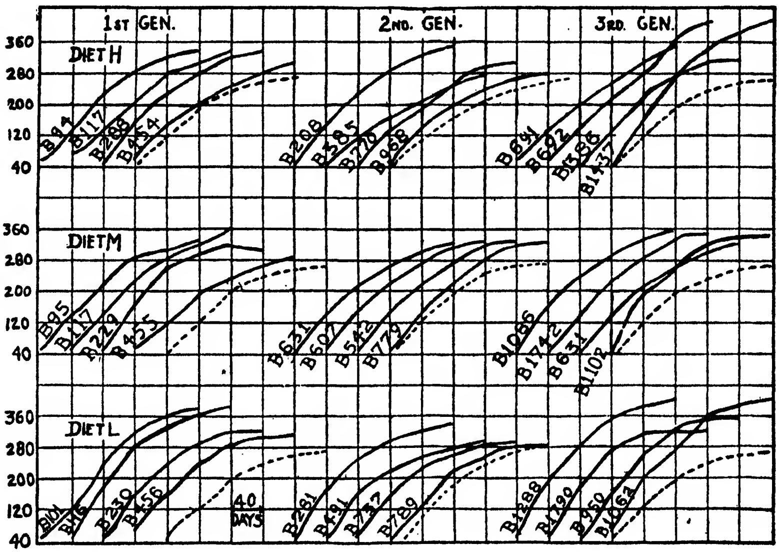

Chart I. Growth of males of three generations from 28th to 228th day of life.

For three generations, the average weight of the young at weaning time was for 336 young on Diet H, 43 gm.; for 217 young on Diet M, 44 gm.; and for 318 young on Diet L, 47 gm.

Reproduction.

Fertility appeared to be normal on all three diets. For three generations the total number of young borne by females up to

1 year of age on each diet was approximately 1100, averaging forty to forty-two young for each of twenty-five to twenty-eight females. The range for each female was practically the same on each diet also, being from ten to eighty young per female on Diet H, four to eighty-four on Diet M, and eleven to ninety-six on Diet L.

From 80 to 85 per cent of the females on each diet bore their first young before the age of 4 months. On Diet L (lowest in flour) there was 10 per cent more mothers bearing young at an early age (under 3½ months) than on either of the other diets. On all three diets, the average number of young borne per mother

TABLE V.

Average Number of Young Borne and Reared in Three Generations on Each Diet.

Generation.	Average No. borne per female bearing young.			Average No. reared by females rearing young.			Per cent of young reared.		
	Diet H.	Diet M.	Diet L.	Diet H.	Diet M.	Diet L.	Diet H.	Diet M.	Diet L.
1st	36	22	35	14	16	16	37	36	45
2nd	45	51	48	15	12	12	33	15	25
3rd	46	46	44	11	11	7	25	24	15

was higher in the second and third generations than in the first, indicating an increase rather than a decrease in fertility. The average age of females at birth of first young was 105 days on Diet H, 103 days on Diet M, and 101 days on Diet L.

Lactation.

The infant mortality was very high and evidently due to failure of lactation. All young were carefully observed for signs of having received milk from their mothers during the first few days of life, and many gave evidence of having received little or none. In three generations on Diet H only 60 per cent of the mothers succeeded in rearing any of their young, on Diet M only 68 per cent, but on Diet L, 75 per cent were able to do so. Of the total young borne, 31 per cent were reared on Diet H, 20 per cent on Diet M, and 26 per cent on Diet L. The number of cases is hardly large enough to make these differences in the three

diets significant. There was an average of forty to forty-two young borne per female, but only thirteen to twenty-three per female reared.

From the above figures it may be seen that while more mothers bore young on Diet L, the infant mortality was no less than on the median diet (M). Furthermore, fertility tended to increase after the first generation, while the percentage of young reared quite consistently decreased, as shown in Table V.

DISCUSSION.

Sherman and Campbell (10) have shown that a diet composed of one-sixth whole milk powder and five-sixths whole wheat or one in which milk furnishes about 20 per cent of the total calories is adequate for growth, but not fully satisfactory for reproduction and lactation, while one in which the milk is increased to one-third of the total ration, or 40 per cent of the total calories, is able to maintain a rat colony through more than twenty generations. Our experiments afford further evidence of the value of milk as a stabilizing factor in the human dietary. The tendency to uniformity in composition of our diets (shown in Table II) in spite of wide variations in the amounts of the different vegetables used, is due very largely to the richness of milk in so many essential substances. The greatest variability in our diets is in the vitamin content. With 25 per cent of the total calories from milk and 8 per cent from a vitamin-bearing fat such as butter, it would seem that fruits and vegetables need not be chosen primarily for the sake of this vitamin, but rather for their contributions of vitamins B and C. Vitamin B, although present in much smaller quantity than in Sherman's Diet 13, has been adequate for superior growth through four generations. It seems probable, however, that it is a limiting factor in lactation, since it has been shown by Sure (11) that a much greater amount is required for suckling the young than for development in early life. This point is being further investigated with the fourth and fifth generations. As vitamin C is not required by the rat in any considerable amount, these studies throw no light on its adequacy in our diets, but a special study with a group of young children leads us to believe that all our diets are adequate for human beings in vitamin C.

Cowgill and some of his coworkers (12) have recently reported studies in which cereal furnished a higher proportion of the total calorie intake, but in their investigations the cereals were whole grain preparations, whole wheat, oatmeal, and yellow corn-meal, and the supplementing part of the rations was compounded with special regard to insuring an adequate diet. In the first series, it was found possible to introduce enough meat residue, dried cooked liver, Osborne and Mendel salt mixture, cod liver oil together with 15 gm. of fresh lettuce daily to obtain normal growth, reproduction, and lactation until the cereal was raised to 84 per cent of the total calories. Above this point there was poorer growth and other evidences of inadequate food. In the second series, eggs, cane molasses, and fresh lettuce were used as supplements with success up to the point where whole grain cereal furnished 64 per cent of the total calories. When the cereal was raised to 80 per cent, the results were more irregular, especially as regards lactation, and at 93 per cent the diet no longer sufficed for rapid growth. The diet at the 80 per cent level is especially interesting to us, because, estimated from average analyses, it has the following composition per 100 calories in comparison with our Diet H with 50 per cent of its calories from flour.

		Protein.	Ca	P	Fe	Vitamin A.	Vitamin B.	Vitamin C.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*units*	*units*	*units*
Cowgill diet:								
Egg	12	4.8	0.039	0.107	0.0016	257–436	50–68	(?)
Molasses	8							
Oatmeal	80							
Lettuce	15							
Diet H		3.7	0.050	0.056	0.0004	240–400	22	1.1

It is evident that with 25 per cent of the total calories from milk, an assortment of common vegetables, furnishing 10 per cent of the total calories, and butter or its equivalent for additional vitamin A contributing from 8 to 12 per cent of the total calories, we have a diet about equal in its power to sustain the race to the Cowgill egg-molasses-lettuce-oatmeal mixture at the 80 per cent level, and one which from the point of view of the well assorted

human dietary has certain advantages over this more restricted ration. This Cowgill diet has the equivalent of about four eggs a day for a child, 2 teaspoons of cod liver oil, and at least two very large heads of lettuce.

The use of whole grain cereals is advantageous to increase the iron and vitamin B content and laxative properties of the diet, without increasing the cost. The freer use of vegetables in a whole grain and milk type of diet will be most valuable for raising the amount of vitamins B and C, increasing the laxative properties, and supplying those concomitant factors which promote economical utilization of iron.

SUMMARY.

Experimental diets with a selection of food materials similar to that found in diets consumed by normal children, but differing in their proportions of cereals (represented by white flour) and vegetables have been fed to albino rats through four generations. A fifth generation has been secured on each diet. In three generations 127 animals have been carried to the age of 1 year and 871 young to the age of 4 weeks.

Food consumption has been very uniform throughout and general health and gains in weight have regularly exceeded the Donaldson "normal" curve.

There were more infertile matings on the diet highest in white flour, but there has been no significant difference in age of mothers at the birth of first young.

On each diet reproduction was better in the second and third generations than in the first, but lactation was poorer in successive generations, as shown by a decline in the per cent of young reared. These experiments furnish further evidence that a diet adequate for excellent growth and reproduction may be inadequate for lactation. It is suggested that the limiting factor is vitamin B, but this point is under further investigation.

BIBLIOGRAPHY.

1. Rose, M. S., Child nutrition and diet, *J. Home Econom.*, 1923, xv, 129.
2. Rose, M. S., and Gray, C. E., The relation of diet to health and growth of children in institutions, in preparation.

3. Sherman, H. C., and Pappenheimer, A. M., A dietetic production of rickets in rats and its prevention by an inorganic salt, *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 193.
4. Hart, E. B., and Steenbock, H., The effect of a high magnesium intake on calcium retention by swine, *J. Biol. Chem.*, 1913, xiv, 75.
5. Haag, J. R., and Palmer, L. S., The effect of variations in the proportions of calcium, magnesium, and phosphorus contained in the diet, *J. Biol. Chem.*, 1928, lxxvi, 367.
6. Sherman, H. S., and Hawley, E., Calcium and phosphorus metabolism in childhood, *J. Biol. Chem.*, 1922, liii, 375.
7. Editorial, Milk calcium for children, *J. Am. Med. Assn.*, 1922, lxxix, 968.
8. Sherman, H. C., and Axtmayer, J. H., A quantitative study of the problem of the multiple nature of vitamin B, *J. Biol. Chem.*, 1927, lxxv, 207.
9. Goldberger, J., and Wheeler, G. A., A study of the pellagra-preventive action of the tomato, carrot, and rutabaga turnip, *Pub. Health Rep., U. S. P. H. S.*, 1927, xlii, 1299.
10. Sherman, H. C., and Campbell, H. L., Growth and reproduction upon simplified food supply. IV. Improvement in nutrition resulting from an increased proportion of milk in the diet, *J. Biol. Chem.*, 1924, lx, 5.
11. Sure, B., Vitamin requirements of nursing young. I. The unusual response of nursing young (*Mus norvegicus-albinus*) to vitamin B administrations: Preliminary report, *J. Am. Med. Assn.*, 1927, lxxxix, 675.
12. Cowgill, G. R., Jones, M. H., Frisch, R. A., and Jackson, G. P., Studies on the effects of abundant cereal intake. I. The use of cereals as the chief source of calories, *J. Am. Med. Assn.*, 1927, lxxxix, 1770; II. The use of supplements other than milk, *ibid.*, 1927, lxxxix, 1930.

STUDIES IN NUTRITION.

II. THE EFFECT OF ADDING EGG TO A DIET ALREADY ADEQUATE.

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In a former paper (1) we described experiments in which diets suitable for children but differing in their proportions of cereals and vegetables had been fed to albino rats through four generations, without finding any significant differences in growth produced by varying the amount of cereal by as much as 50 per cent. In these diets one-fourth of the calories was derived from milk and from 10 to 20 per cent from an assortment of common vegetables.

In studies with a group of young children, we found that the addition of an egg a day to a diet which appeared to be adequate resulted in improvement in general health and in the hemoglobin content of the blood (2).

To extend this study over a longer period of life than is possible with human beings we have had recourse to use of experimental animals. We have taken our median diet in the former series, having 37.5 per cent of its calories in the form of white flour, and added to it sufficient egg to represent one egg a day in the diet of a child or 3 per cent of the total calories. The nutritive values of the two diets are shown in Tables I and II.

The laboratory procedures were the same as in the preceding paper. Food consumption was regular, and the average number of calories eaten per gm. of rat per day did not differ markedly from the consumption on the other diets. Calculated to gains in weight per 1000 calories and compared with gains on Sherman's

Diet 13,[1] the results are as follows: on Diet E, 75 ±1.0 gm.; on Diet M, 70 ±1.0 gm.; and on Diet 13, 77 ± 0.6 gm.[2]

The gains in weight of the young from the 28th to 56th day of life for three generations were greater, both for males and females, than on any of the other diets, as shown in Table III.

In every case the difference between the means is significant in favor of Diet 25. Between Diet E and Diet H, the means with

TABLE I.

Composition of Diets.

	Weights of dry ingredients per 100 gm.		Total calories from each food.	
	Diet M.	Diet E.	Diet M.	Diet E.
	gm.	*gm.*	*per cent*	*per cent*
Flour	43.23	43.33	37.5	37.5
Milk	20.04	20.09	25.0	25.0
Dextrin	9.67	9.70	9.5	9.5
Butter substitute	5.43	4.61	10.0	8.5
Meat residue	3.30	1.65	3.0	1.5
Peas, dried	2.16	2.16	2.0	2.0
Potatoes	11.29	11.31	9.8	9.8
Carrots	2.52	2.53	2.6	2.6
Spinach	0.24	0.25	0.3	0.3
Tomato	0.90	0.90	0.2	0.2
Onions	0.22	0.23	0.2	0.2
Egg		2.25		3.0

their probable errors are for males, 18 ±2.4; for females, 10 ±1.3; between Diet E and Diet M, for males, 12 ±2.6; for females, 10 ±1.4; between Diet E and Diet L, for males, 7 ±2.6; for females, 10 ±1.4.

The promise of these early gains was carried out in later life, as will be seen from the growth curves of the males in each generation carried to the age of 1 year (Chart I).

[1] Private communication. Diet 13 consists of one-third whole milk powder and two-thirds ground whole wheat plus sodium chloride equal to 2 per cent of the weight of the wheat.

[2] Private communication.

This diet not only induced better growth in the young, but also earlier maturity of the females, as shown in Table IV.

The number of young borne per female was practically the same as on three corresponding diets without the egg (Diet M), but the number of young reared by all females bearing young was considerably higher, averaging sixteen per female on Diet E and nine on Diet M. The number of young reared per female rearing young was higher for the egg diet, averaging twenty per female or 42 per cent on Diet E as against sixteen per female or 26 per cent on Diet M, 11 per cent on Diet H, and 26 per cent on Diet L. One mother reared 98 per cent of her offspring, a feat accomplished by none on

TABLE II.

Nutritive Value per 100 Calories.

	Diet M.	Diet E.
	gm.	*gm.*
Protein	3.7	3.6
Fat	2.6	2.7
Carbohydrate	15.4	15.4
Calcium	0.051	0.053
Phosphorus	0.056	0.060
Iron	0.00048	0.00052
	units	*units*
Vitamin A	333–621	365–653
" B	22	24
" C (cooked food)	2	2

TABLE III.

Average Gains in Weight from 28th to 56th Day of Life.

	Diet E.		Diet H.		Diet M.		Diet L.		Diet 13.
	No. of cases.		No. of cases.		No. of cases.		No. of cases.		
		gm.		*gm.*		*gm.*		*gm.*	*gm.*
Males	39	87 ±1.6	22	69 ±1.8	20	75 ±2.0	24	80 ±2.1	82.9 ±0.8*
Females	58	65 ±0.8	32	55 ±1.0	32	55 ±1.2	36	55 ±1.2	62.4 ±0.6*

* Private communication.

any of the other diets. The improved lactation is also shown by the fact that the percentage of females bearing but not rearing young was lower than on any other diet, being only 20 per cent for Diet E as against 32 per cent on the corresponding diet without egg (Diet M), and 40 per cent on the diet highest in flour (Diet H).

Sherman and MacLeod found that the seemingly simple dietary change from one-third whole milk powder and two-thirds whole wheat to one-third skim milk powder and two-thirds whole wheat

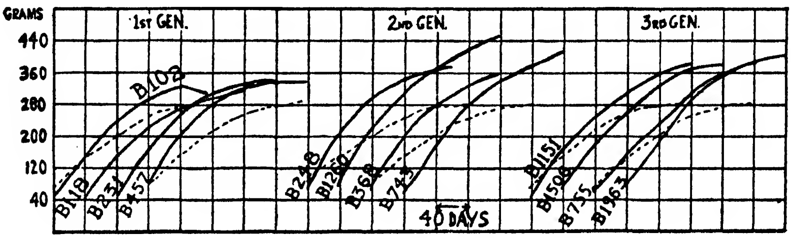

CHART I. Growth of males of three generations on egg diet from 28th to 228th day of life.

TABLE IV.

Age of Females at Birth of First Young.

Diet.	No. of females.	Age at birth of first young.
		days
H	32	105 ±2.8
M	33	103 ±3.5
L	32	101 ±2.3
13		116 ±3.0
E	49	94 ±1.0

produced measurable differences in growth, reproduction, and longevity (3).

We have found that an apparently smaller change—the incorporation of egg equal to only 3 per cent of the total calories—has had a similar influence on the life histories of our rat families. Starting with a diet already proved to be efficiently utilized and adequate for growth, we were surprised to find the egg diet not only more economically used for growth, as shown by the gains in weight per 1000 calories consumed, but also resulting in greater growth of

the young, as indicated by weight gains for males during the 1st month (corresponding to 2½ years in the life of a child) which not only exceeded gains on any of our three diets without egg but even the excellent growth record of Sherman and Campbell's colony on Diet 13. Our experience again parallels Sherman and MacLeod's in the earlier maturity of the females, in the larger number bearing young, and in the higher proportion of the offspring reared. These differences can scarcely be accounted for by any quantitative changes in the diet which we have been able to measure. The differences between any two of our three no-egg diets are apparently as great as the differences between the median diet and its counterpart with added egg. The amount of vitamin B is slightly higher, and perhaps this is enough to make the difference between good

TABLE V.

Differences in Percentage of Hemoglobin with Age.

Diet.	6 wks.		2½ mos.	
	2nd generation.	4th generation.	2nd generation.	4th generation.
	per cent	*per cent*	*per cent*	*per cent*
A	67 ±1.7	64 ±1.2	83 ±0.6	78 ±1.1
L	72 ±1.0	67 ±1.4	84 ±1.0	84 ±1.1
E	66 ±1.7	60 ±1.2	81 ±1.6	82 ±1.2

and poor lactation. The protein is no higher, which confirms our opinion that protein was not the limiting factor in any of our diets. It is possible that the egg supplies an additional factor necessary to the best lactation (4), and this might also account for the better growth of the young.

As we found the egg to improve children's blood in regard to its hemoglobin percentage and erythrocyte count we thought that the good effects on our rats might be due to a similar improvement. We therefore made hemoglobin and erythrocyte determinations on our rats at the ages of 6 weeks and 2½ months in the second and fourth generations on the egg diet, the one highest in flour (Diet H), and the one lowest in flour (Diet L). To our surprise we found no significant difference between the two generations, but we did find a difference due to age in the percentage of hemoglobin in accord with the findings of Williamson and Ets (5), as shown in Table V.

These findings confirm our opinion that all of our diets have been adequate in iron for the rat. Before we can make comparisons with human requirement, we must find out what the minimum requirement of the rat is. Further studies are in progress.

SUMMARY.

Three diets suitable for young children, differing from each other only in the relative proportions of cereal food and vegetables, were all able to induce good growth in young rats through four generations; also a good degree of fertility but poor lactation. To the one of these diets having the median proportion of cereal (37.5 per cent of the total calories) and of vegetables (15 per cent of the total calories) was added egg in amount equal to 3 per cent of the total calories or 2.25 per cent of the total dry weight, and corresponding to an egg a day in the diet of a growing child. The egg diet proved equal to any of the others in the number of young borne and the weight of the young at the age of 28 days; and superior to all the other diets (1) in the growth of the young from the 28th to the 56th day of life, (2) in the age at which the females matured as shown by the age of the mothers at the time of the birth of their first litter, (3) in the number of females bearing young, (4) in the capacity of the mothers to suckle their young, as shown by the greater number of young reared compared with the total number produced. The superiority of the egg appears not to be due, in case of the rat, to its effect on the hemoglobin content of the blood. Hemoglobin has been found to vary with age rather than diet, being higher at 2½ months of age than at 6 weeks. These investigations afford further evidence of the possibility of improving a diet which is adequate for growth, and the usefulness of animal experimentation in finding optimum food combinations. They show, also, the importance of milk and eggs as staple foods for the production of a vigorous race.

BIBLIOGRAPHY.

1. Rose, M. S., and McCollum, E. L., Studies in nutrition. I. Growth, reproduction, and lactation on diets with different proportions of cereals and vegetables, *J. Biol. Chem.*, 1928, lxxviii, 535.
2. Rose, M. S., The influence of prolonged administration of egg upon the hemoglobin content of children's blood, *J. Biol. Chem.*, 1926, lxvii, p. xx.

3. Sherman, H. C., and MacLeod, F. L., The relation of vitamin A to growth, reproduction and longevity, *J. Am. Chem. Soc.*, 1925, xlvii, 1658.
4. Evans, H. M., and Burr, G. O., A new dietary deficiency with highly purified diets. III. The beneficial effect of fat in the diet, *Proc. Soc. Exp. Biol. and Med.*, 1927–28, xxv, 390.
5. Williamson, C. S., and Ets, H. N., The effect of age on the hemoglobin of the rat, *Am. J. Physiol.*, 1926, lxxvii, 480.

OBSERVATIONS ON THE RENNIN COAGULATION OF MILK.

THE EFFECT OF HIRUDIN, OF HEPARIN, OF CEPHALIN, AND OF FAT REMOVAL.

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The rennin coagulation of milk has been described as resembling in many ways the fibrin ferment coagulation of blood (1). While subsequent investigation has shown this resemblance to be more apparent than real, it is not impossible that one of the chain of steps involved in the complex phenomenon of blood coagulation may be analogous to the simpler mechanism of milk coagulation by rennin. Thus Epstein and Rosenthal (2) have reported that pancreatic rennet free from lipoid greatly hastens the coagulation of blood. Crude pepsin solutions on the contrary, though having milk-coagulating power, they found delayed blood coagulation. However, the quantities of pepsin used were rather large so that it is within the range of possibilities that in their experiments enough proteolysis may have occured to mask any effect upon the clotting time. There are apparently enough analogues between fibrin ferment and rennin to suggest that possibly some of the agents that inhibit or favor fibrin ferment action may have analogous effects upon rennin action. To inquire into some of these possibilities was the purpose of the present investigation.

Hirudin and heparin, as blood coagulation-inhibiting agents, and cephalin, as a blood coagulation-favoring agent, at once suggest themselves for such an investigation as this. The effect of these substances upon rennin action does not seem to have been investigated. Hirudin is prepared from leeches and has a powerful action in preventing the coagulation of blood (3) when added *in vitro* or injected intravenously. Heparin was prepared from the liver by Howell and Holt (4). The mechanism of its action

is quite different from that of hirudin. Cephalin was shown by Howell (5) to be the zymoplastic cell substance, but Mills (6) has presented evidence in support of the view of Woolridge that tissue coagulins are protein-lipoid combinations.

To determine the effect of cephalin upon rennin coagulation of milk it was necessary, obviously, to free both the rennin solution and the milk from lipoid, since it is not known whether either is free from cephalin. This necessitated a study of the effect of fat solvents upon rennin and upon milk. Since it was found impracticable to extract the fat from whole milk with immiscible solvents because of the formation of emulsions, skim milk had to be used. This in turn necessitated investigation of the effect of skimming upon rennin coagulation of milk. Hence as a by-product of the investigation observations upon these phenomena are presented in the present paper, for the literature contains contradictory statements concerning the effects of fat solvents and of skimming upon milk coagulation.

There is a large literature dealing with the effect of antiseptics upon milk; but only that portion of it dealing specifically with the effect of ether, alcohol, chloroform, benzene, or petroleum ether upon rennin need be considered here.

Glaessner (7) found that alcohol and ether destroy prochymosin while chloroform and toluene do not. He did not study the effects of these substances upon the enzyme itself but states that on the whole the proenzymes behave in the presence of these agents like the enzymes that are derived from them. The inference then is that he believed alcohol and ether destroy chymosin (rennin) while chloroform and toluene do not.

Fuld (8) states that ether added to milk in no way inhibits rennin coagulation. Doyon (9) found that ether and alcohol considerably favor the coagulation of milk by rennin, whereas chloroform and "benzine" have no favorable influence.

Concerning the effect of the presence of the fat and lipoid of milk on rennin coagulation opinions also differ. Bräuler (10) removed the fat globules from milk by adding aqueous alkali and extracting. From such defatted milk casein could be precipitated in the typical way by the addition of acid; but the milk could not be coagulated with rennin. It was too alkaline. Its neutralization resulted in too great dilution. Kreidl and Lenk (11) found that different samples of milk of different fat content behave differently in the presence of rennin. A series of market milks and creams was examined with fat contents ranging from 1 to 30 per cent. When undiluted there was but little difference. When diluted with an equal volume of water rennin coagulated most slowly those containing the

most cream, but most rapidly those containing the least. Skim milk always coagulated soonest and cream last.

Mayer (12) found the fat content of milk without effect upon rennin action. He skimmed the milk by gravity. Lezé and Hilsont (13) reached the opposite conclusion. They experimented upon mixtures of skim milk and cream. Davarda (14), although he observed some retardation in skim milk, nevertheless agreed with Mayer. Höft (15) found as the average of nineteen experiments upon separator-skimmed and whole milk a coagulation time of 205.7 seconds for the former and 189.3 seconds for the latter. The skim milks were usually a trifle less acid than the whole milks as determined by titration, but Höft doubted that this had anything to do with his observations. It is obvious, therefore, that there is far from agreement concerning the effect upon rennin coagulation of removal of the fat either by solvents or by different mechanical methods.

Of hirudin two samples were used in the present investigation. One had been purchased in Germany in 1910. After a portion had been used at that time the remainder, a few decigrams, was sealed in a small unevacuated glass tube and kept thus at ordinary temperatures. In the winter of 1924–25, when the tube was opened and the preparation used for the present investigation, it was still active, though by no means as much so as when it was fresh. The other sample of hirudin was purchased from the firm of Schuchardt in Görlitz, Germany, in the fall of 1926. It was quite active.

Seven different samples of heparin were used, all obtained from Hynson, Westcott and Dunning of Baltimore. The manufacturers very kindly informed us that these "represent different preparations, but were prepared by essentially the same process. Different lots of Heparin, however, require more or less purification to bring them up to the required standard of 1 milligram preventing the coagulation of 5 cc. of blood within twenty-four hours. Sometimes it is necessary to re-process the product over and over again to eliminate contamination and related substances. It is a very difficult and tedious procedure."

We desire to thank Dr. H. A. B. Dunning of the firm of Hynson, Westcott and Dunning for this information as well as for other courtesies. The samples bore the numbers 46, 102, 23, 42, 24, 48, 49. Of Preparation 24 two different samples were secured, one obtained directly from the manufacturer, the other from Professor Hanzlik, of Stanford University, which he had received from the manufacturer and had been using in his own research.

The writers did not themselves test the blood coagulation-inhibiting power of these preparations. However, Professor Hanzlik very kindly tested Preparations 48 and 49 and found them practically equally active and up to the standard claimed for them by the manufacturers. We desire to take this occasion to thank him for this and other courtesies.

Since according to the statement of the manufacturers heparin preparations are admittedly mixtures, and since the procedure of preparation is not exactly the same for any two preparations, it is altogether probable that the different heparin preparations, though of the same anticoagulating strength, may yet have contained adventitious material in different proportions. The significance of this possibility will become clear later in this paper.

The cephalin used in this investigation was obtained from Hynson, Westcott and Dunning. Experiments were also made with "thromboplastin," a blood coagulation-favoring preparation made by E. R. Squibb and Sons.

The rennin used was prepared by the Marschal Dairy Laboratory of Madison, Wisconsin, from calves' stomachs. It contained as preservatives, sodium chloride, boric acid, and thymol. Two different samples were secured. These were used diluted with water in proportions varying from 1:120 to 1:325. In these dilutions the percentage of salt, boric acid, and thymol was very small.

The method employed to determine the rate of coagulation is as follows: A definite amount of milk was placed in a test-tube; the rennin was added and the tube inverted twice to mix the solution, at which moment the time was noted. The tubes were immediately placed in a water bath at 38°. From time to time the tubes were tilted to note the state of the milk. When the milk became so gelatinous that, when the tube was slanted, it did not flow characteristically as fluid milk, the time was again noted and this was taken as the coagulation time.

Neither preparation of hirudin examined by this method showed any effect whatever upon the coagulation time even when the amount used in the milk was far in excess of the amount necessary to inhibit completely the coagulation of an equal volume of blood. In some experiments the hirudin in aqueous solution was added to the milk and the mixture allowed to stand for 10 minutes before

the addition of the rennin. In others the hirudin either in solution or solid was added to the rennin solution and allowed to stand for 10 minutes before the rennin was added to the milk. In neither case was any retarding or accelerating effect noted. The rennin solutions used were in some cases unmodified; in others they had been rendered fat-free in the manner described below.

The effect of the heparin varied from preparation to preparation. Of the seven preparations used two caused very great retardation of coagulation; one, moderate but distinct retardation; the remaining four caused retardation so slight that it can be given no great weight. In Table I are given the results of typical experiments with two preparations, one very active, the other but slightly so. The coagulation times in the different sets of experiments differ partly because different samples of milk were used in the different sets, partly because different rennin preparations were used in different amounts, partly because in some

TABLE I.

Effect of Heparin on Rennin Coagulation Time at 38°.

Heparin preparation No.	Milk.	1 per cent NaCl.	Water.	Heparin.	Fat-free rennin.	Coagulation time.
	cc.	*cc.*	*cc.*	*mg.*	*cc.*	*min.*
46	10	3		0	0.5	55
	10	3		3	0.5	No coagulation, 150.
	10		3	0	0.5	77
	10		3	2	0.5	No coagulation, 150.
	10		3	3	0.5	" " 150.
102	10	3		0	0.3	43
	10	3		2	0.3	43
	10	3		3	0.3	43
102	10		2	0	0.2	32
	10		2	4	0.2	40
102	10		2	0	0.3	19
	10		2	4	0.3	22
	10		2	8	0.3	28
102	10		2	0	0.3	20
	10		2	16	0.3	22

experiments the rennin, heparin, and milk were mixed practically simultaneously, while in others the heparin was added to the rennin solution and allowed to stand with it for different periods before it was added to the milk.

It seems that the inhibiting action of active heparin is greater if it is first allowed to act upon the rennin than if it acts upon the milk before the rennin is added. This is shown by the experiments recorded in Table II. Indeed unless the heparin is very active it may show no effect at all if it is added to the milk, the

TABLE II.

Effect of Heparin on Activity of Rennin when Allowed to Stand in Contact with It for Different Periods at Room Temperature.

Experiment No.*	Time of contact.	Coagulation time.
	min.	*min.*
657	10	34
658	10	48
659	20	33
660	20	65
661	30	34
662	30	70
663	40	33
664	40	86

* 2 mg. of heparin Preparation 23 added only in even numbered experiments per 0.2 cc. of fat-free rennin. Raw milk 5 cc.

mixture allowed to stand, and then the rennin added. Apparently those heparin preparations which show inhibiting effects act upon the rennin rather than upon the milk.

Examination of Table I suggests that the action of heparin tends to become more pronounced with rising dosage. This is brought out by the following experiment.

Heparin Preparation 23; 0.2 cc. fat-free rennin solution 1:120; 10 cc. raw whole milk.

Heparin.	Coagulation time.
mg.	*min.*
0	59
2	72
4	120
6	145

The experiments of Table I in which the larger amounts of heparin were used show some indication that the heparin effect becomes more noticeable the longer the coagulation time. This is only what is to be expected since, if the action of heparin is on the rennin, the action would be more pronounced when the rennin action is weak; that is, when relatively little rennin is present. This is brought out by the following sets of experiments in which

TABLE III.

Raw milk.	Rennin.	Heparin.	Coagulation time.	
cc.	*cc.*	*mg.*	*min.*	
5	0.2		16	16
5	0.2		16	
5	0.2	0.2	18	18
5	0.2	0.4	21	19
5	0.2	0.6	23	22
5	0.2	0.8	24	25
5	0.5		6	
5	0.5	0.5	6	
5	0.5	1.0	8	
5	0.5	1.5	9	
5	0.5	2.0	10	
10	1.0		10	
10	1.0	4.0	12	
10	0.8		10	
10	0.8	4.0	15	
10	1.0		10	
10	1.0	4.0	14	
5	0.2		16	
5	0.2		17	
5	0.2	0.8	19	
5	0.2	0.8	24	

the rennin was allowed to remain in contact with the heparin for 2 hours before being added to the milk.

The rennin stock solution was 2 years old. It had been exhausted with ether (see below) and preserved by one-thirtieth of its volume of pure ether in addition to the traces of boric acid and thymol it contained. For the experiments it was diluted 1:120. The data are given in Table III.

As a check upon these experiments, exactly similar ones were

made with, however, fresh rennin solution which had not been treated with ether. The results were as follows:

Raw milk.	Rennin.	Heparin.	Coagulation time.	
cc.	*cc.*	*mg.*	*min.*	
5	0.2		16	16
5	0.2	0.8	20	25
5	0.2	0.8	23	23
5	0.2	0.8	24	
5	0.2		16	16

A great many more experiments were made besides those recorded above, but they are not reported here since they add no new facts. As in the above experiments, some preparations caused only a few minutes delay when allowed to stand in contact with rennin for some time before being mixed with milk and these retardations were noticeable principally when the dosage of rennin was so small that the coagulation time of the controls without the heparin was at least 10 minutes. Some heparin preparations under these circumstances caused a retardation of some hours.

Since all these heparin preparations had apparently the same power of inhibiting blood coagulation but differed so markedly in power of inhibiting milk coagulation, it is probable that the antirennin and the antifibrin ferment action of heparin have nothing to do with one another. The simplest explanation of the phenomena here recorded is that heparin preparations may contain at least two substances, one inhibiting the action of blood coagulation, the other destroying or neutralizing rennin. The preparations examined in the present investigation were probably uniform in regard to their content of blood coagulation-inhibiting substance but very variable in regard to their content of rennin-neutralizing substance. Very probably in the process of purifying the different preparations the antirennin, if this is what it is, was removed in varying degree in the different preparations. Concerning the character of the rennin-neutralizing substance there is as yet no information. It may be nothing more than the antirennin of the liver, for antirennin is said to be very widely distributed in the tissues (16). It would be of interest as bearing upon the disputed question of the possible identity of rennin and

pepsin to see whether heparin or the antirennin of the liver inhibits the proteolytic action of pepsin.

As indicated above, preliminary to study of the effect of cephalin, milk and rennin solutions had to be freed from fat and lipoid.

TABLE IV.

Solvent used.	Solvent.	Whole milk.	Rennin.	Water.	Coagulation time.
	cc.	*cc.*	*cc.*	*cc.*	*min.*
Ethyl alcohol, 96 per cent by volume.	0	10	1	3.0	21
	1.1	10	1	1.9	87
	1.2	10	1	1.8	317
	1.3	10	1	1.7	No coagulation, 360.
	1.4	10	1	1.6	" " 360.
Chloroform.	0	10	1	3.0	22
	0.2	10	1	2.8	48
	0.3	10	1	2.7	55
	0.4	10	1	2.6	85
	0.5	10	1	2.5	No coagulation, 180.
Benzene.	0	10	1	3.0	20
	0.1	10	1	2.9	23
	0.2	10	1	2.8	26
	0.5	10	1	2.5	31
	1.0	10	1	2.0	39
	2.0	10	1	1.0	48
	3.0	10	1	0	52
Petroleum ether.	0	10	1	5.0	27
	0.1	10	1	4.9	27
	0.2	10	1	4.8	28
	0.5	10	1	4.5	29
	1.0	10	1	4.0	30
	2.0	10	1	3.0	30
	3.0	10	1	2.0	31
	4.0	10	1	1.0	31
	5.0	10	1	0	32

Extraction of fat and lipoid from the rennin solutions presented no technical difficulties. The undiluted solution was merely extracted repeatedly with purified ether until the ether extract left but a trace of residue which was completely soluble in water. It

was deemed important to purify the ether since it may contain alcohol and aldehyde. U. S. P. ether was washed repeatedly with distilled water and then dried over solid NaOH for 3 days. It was then distilled over solid NaOH to polymerize any aldehyde.

The rennin solution thus extracted and still containing such an amount of ether as remained dissolved in it, coagulated milk in exactly the same time as it did when not extracted and therefore containing no ether. Since the amount of rennin solution added to the milk (10 cc.) was never over 1 cc., the amount of ether in the milk rennin solution mixture was very minute. It was obvious that neither the removal of the ether-soluble lipoid material from rennin solutions nor the presence of a trace of ether affected in the slightest degree the coagulating power of the solutions. Accordingly, in the course of the work the dilute rennin solutions were preserved with one-thirtieth of their volume of pure ether. At laboratory temperatures they preserved their activities practically unaltered for more than 2 weeks. In one case an undiluted rennin solution was kept for a year without material change in its activity.

The removal of fat and lipoid from milk presented more difficulty. The first step was to determine whether ether in itself changed the coagulation time of milk. It was found that saturating milk with ether and then removing it by evaporation *in vacuo* with an air current bubbling through a capillary which dipped into the milk did not in the least change the coagulation time.

It was next determined whether or not the presence of ether changes the coagulation time. The results were as follows:

Whole milk.	Rennin.	Water.	Ether.	Coagulation time.
cc.	*cc.*	*cc.*	*cc.*	*min.*
10	1	3.0	0	22
10	1	2.9	0.1	32
10	1	2.8	0.2	37
10	1	2.5	0.5	64
10	1	2.0	1.0	No coagulation, 345.
10	1	1.0	2.0	" " 345.
10	1	0	3.0	" " 345.

It is obvious, therefore, that if ether is to be used to free milk from lipoid material by ether extraction, the ether must subse-

quently be removed thoroughly before the milk can be used for the tests. It was deemed advisable, therefore, to see whether some other fat solvent which would not inhibit rennin coagulation could not be used. Alcohol, chloroform, benzene, and petroleum ether were therefore tested. The results are shown in Table IV.

It is seen that petroleum ether has the least effect, yet its use, as well as that of the other solvents, to remove the fat proved impracticable because of the formation of emulsions. Therefore recourse was had to milk skimmed in a separator. For this purpose a small capacity, hand-operated, standard type separator was used. To bring about a good separation in a device of this kind, it has been found necessary to warm the milk to about 32° before it is run through the machine. As it was necessary to learn whether milk skimmed in this way had the same coagulation time as the unskimmed milk, some of this warmed milk was kept apart, not skimmed, and used as a control.

When the coagulation time of the skim milk was compared with that of the control, it was found that the skim milk coagulated more slowly than the whole milk, all conditions being kept as nearly alike as possible. The difference averaged 40 per cent. Even when the milks were kept for a number of days and tested each day, the result was the same. The following is one of a considerable number of sets of such experiments which were performed. The age of the milk was assumed to be zero at the time of the skimming. Between observations the milks were kept on ice.

Whole milk.	Separator-skimmed milk.	Age.	Rennin.	Coagulation time.
cc.	*cc.*	*hrs.*	*cc.*	*min.*
10		0	1	16
	10	0	1	22
	10	0	1	22
10		24	1	13
	10	24	1	18
10		48	1	12
	10	48	1	17

Since it was found possible to remove the small amount of fat remaining in separator-skimmed milk by extraction with solvents,

the effect of ether extraction on the coagulation time of such skim milk was next studied.

20 cc. portions of the skim milk were extracted in a separatory funnel four times with 50 cc. portions of purified ether. The traces of ether were removed from the milk by vacuum distillation with an air current as before. It was found that such extracted skim milk coagulated slightly more slowly than the same skim milk unextracted. The following is a typical experiment.

Milk.	Amount of milk.	Rennin.	Coagulation time.
	cc.	*cc.*	*min.*
Whole	10	1	18
Separator-skimmed	10	1	26
" extracted	10	1	29

The method finally adopted to free milk from lipoid was to skim it as above, then to extract it with ether as above, and finally to remove the ether as above.

To test the effect of cephalin, 10 cc. of such extracted skim milk were triturated with 0.4 gm. of cephalin in a mortar. Then 1 cc. of the rennin solution was added. In this case as in all the experiments reported above the rennin solution had been freed from lipoid by extraction with ether. No influence whatever on the coagulation time could be noted. This was also true for those experiments not here reported in which the rennin solution had not been freed from lipoid. When the same experiment was performed with whole milk, there was also no effect. The experiments were repeated with thromboplastin with the same negative result. So far as these experiments go they indicate that cephalin, which so greatly favors blood coagulation, is without effect upon rennin coagulation. The experiments are not perfectly conclusive since it has not been proved that coagulants like those of tissues, if present in milk or rennin, were completely removed by the procedures used.

The effect of ether and other fat solvents upon the coagulation time of rennin has been attributed to the surface tension-lowering effect of these substances. To test this hypothesis the effects of saponin and of soap, both of which greatly lower surface tension, were examined. The saponin used was a commercial preparation.

It was wholly without effect even when 30 mg. were added to 10 cc. of whole milk. Soap was used as a 1.25 per cent aqueous solution of Castile soap carefully neutralized to phenolphthalein with 0.1 N NaOH. It delayed coagulation quite considerably but it is doubtful that this is due to surface tension lowering since the addition of a small amount of 1 per cent $CaCl_2$ solution completely prevented the unfavorable action of the soap. Most probably therefore the action of the soap was due to the removal of calcium ions, though it is also possible that change in the hydrogen ion concentration may have played a rôle. Some of the experiments with soap gave the following results.

Whole milk.	Rennin.	Water.	Soap solution.	$CaCl_2$	Coagulation time.
cc.	*cc.*	*cc.*	*cc.*	*cc.*	*min.*
10	1	3.0	0		22
10	1	0.8	2.2		88
10	1	0.5	2.5		102
10	1	0.3	2.7		117
10	1	0	3.0		135
10	1	3.5	0		24
10	1		3.0	0.5	11
10	1		3.0		138

As already stated, Höft (15) suggested that perhaps the slower coagulation of separator-skimmed milk is due to its somewhat lower titratable acidity. This change in acidity could not be verified in the present investigation. It has been suggested to the writers by Dr. L. L. Van Slyke that possibly the effect of separator skimming is due to the removal of potential calcium ions in the slime. Now Bahlman (17) has shown that the calcium content of separator slime is but 0.44 per cent. Therefore, since the dry slime is about 0.1 per cent of the weight of the milk, the amount of calcium removed in it is entirely too small to account for the effect. Attempts were made by the writers to return the slime to the milk and also to return the cream that had been separated, in order to see whether thereby the coagulation time would be changed back to its original value. The results were unsatisfactory since it was found impossible to mix or dissolve either in the skim milk so as to bring it back to its condition before skimming.

Since Mueller (18) found that a delay of coagulation could be induced by merely shaking milk for a time, and since it is generally known that many colloids are easily altered by physical treatment, it was thought that possibly the violent agitation caused by the separator was the important factor. If this be so, then skimming milk without agitation should not change its coagulation time.

Accordingly, milk was skimmed in two ways: by gravity and by centrifugation in tubes.

100 cc. of whole milk were placed in a separatory funnel and allowed to stand on ice for about 16 hours to permit the cream to rise; a quantity of the same milk was similarly kept on ice to be used for a control. The milk below the cream layer in the funnel was then drawn off. The butter fat content of the whole milk and the gravity-skimmed milk was determined by the Röse-Gottlieb method and was found to be 3.91 and 0.88 per cent respectively. The two milks showed no difference whatever in coagulation time. To skim milk by centrifugation, milk was placed in the glass tubes of an ordinary electrically driven laboratory centrifuge. After centrifugation the lower part of the milk in the tubes was pipetted off and again centrifuged. This was repeated several times. The milk then appeared thoroughly skimmed but fat was not determined. The coagulation time had not been changed.

These experiments, together with the fact that in the separator slime but minute traces of calcium are removed, would seem to indicate that it is the violent agitation by the separator that accounts for the longer coagulation time of skim milk. Unfortunately, there is a serious objection to this hypothesis. It is the effect of "clarifying" milk. Some milk was run through a clarifying machine and its coagulation time remained quite unchanged thereby. This machine operates on the same principle as a separator only it is so arranged as to remove the slime as well as any other solid matter such as dust or dirt without removing the cream. It certainly subjects the milk to much agitation.

At any rate, while it is not possible to reach a final conclusion concerning the cause of the longer coagulation time of separator-skimmed milk, it seems possible that the lack of agreement of previous investigators in regard to the effect of skimming upon

coagulation time is due at least in part to the manner of skimming. In future investigations the importance of this factor will have to be borne in mind.

SUMMARY.

Neither hirudin nor cephalin exerts any influence on the rate of coagulation of milk by rennin.

Heparin may or may not delay the coagulation of milk by rennin. Some preparations delayed notably, others delayed but slightly or not at all; yet all had about the same effect upon blood coagulation. Therefore, heparin preparations which delay rennin coagulation must be regarded as mixtures containing both blood coagulation-inhibiting and milk coagulation-inhibiting material. There is no evidence that the two effects have anything to do with one another.

Heparin preparations which delay rennin coagulation of milk act upon the rennin rather than upon the milk.

Extracting the fat and lipoid from a rennin solution with pure ether and allowing the solution to remain for a considerable time saturated with ether does not affect its coagulating power.

Pure ether, ethyl alcohol, and chloroform inhibit the rennin coagulation of milk when present in sufficient quantity. Benzene delays coagulation slightly; petroleum ether still less. The effect is probably not due solely to lowering of surface tension for saponin is without effect. A neutralized soap solution greatly delays coagulation but the effect is probably due mainly to the removal of calcium ions, since the addition of small amounts of calcium chloride prevents the effect.

Milk skimmed by a separator coagulates more slowly than milk skimmed by gravity.

BIBLIOGRAPHY.

1. Arthus, M., *Compt. rend. Soc. biol.*, 1893, v, 435.
2. Epstein, A. A., and Rosenthal, N., *Proc. Soc. Exp. Biol. and Med.*, 1921, xix, 79.
3. Haycraft, J. B., *Arch. exp. Path. u. Pharmakol.*, 1884, xviii, 209. Franz, F., *Arch. exp. Path. u. Pharmakol.*, 1903, xlix, 342. Bodong, A., *Arch. exp. Path. u. Pharmakol.*, 1905, lii, 242.
4. Howell, W. H., and Holt, E., *Am. J. Physiol.*, 1918, xlvii, 328. Howell, W. H., *Am. J. Physiol.*, 1923, lxiii, 434.

5. Howell, W. H., *Am. J. Physiol.*, 1912, xxxi, 1.
6. Mills, C. A., *J. Biol. Chem.*, 1920, xlvi, 135.
7. Glaessner, K., *Beitr. chem. Physiol. u. Path.*, 1902, i, 19–20.
8. Fuld, E., *Ergebn. Physiol.*, *1. Abt.*, 1902, i, 468.
9. Doyon, M., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 918.
10. Bräuler, R., *Arch. ges. Physiol.*, 1910, cxxxiii, 519.
11. Kreidl, A., and Lenk, E., *Biochem. Z.*, 1914, lxiii, 151.
12. Mayer, A., *Landw. Versuch.-Sta.*, 1881, xxvii, 247.
13. Lezé, R., and Hilsont, E., *Milch-Ztg.*, 1894, xxiii, 362.
14. Davarda, A., *Landw. Versuch.-Sta.*, 1896, xlvii, 401.
15. Höft, H., *Milchwirtschaft. Zentr.*, 1908, iv, 489.
16. Effront, J., Biochemical catalysts in life and industry, translated by Prescott, S. C., New York, 1917, 88.
17. Bahlman, C., *Am. J. Pub. Health*, 1916, vi, 855.
18. Mueller, W., *Biochem. Z.*, 1912, xlvi, 94.

THE DIGITALIS GLUCOSIDES.

I. DIGITOXIGENIN AND ISODIGITOXIGENIN.

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(Received for publication, May 3, 1928.)

Our investigations on the structure of strophanthidin were begun a number of years ago with the hope that the information obtained would be useful in similar studies with other members of the pharmacological group of substances to which it belongs. As previously presented,[1] the generally suspected close chemical relationship of many of these substances has been made more apparent by observations on their parallel behavior toward Tollens' reagent and sodium nitroprusside. These results have suggested that the aglucones of this group are $\Delta^{\beta,\gamma}$-lactones like strophanthidin in which the double bond may be found within or without the lactone ring. Attempts however to prove directly the presence of the $\Delta^{\beta,\gamma}$-lactone group in such substances as ouabain and the digitalis aglucones by the preparation of an oxime or a semicarbazone after gentle saponification of their lactone groups were without success.[2] A similar result was also obtained with certain strophanthidin derivatives. Fortunately, however, a means has now been found to accomplish this object, based upon the method recently used in the isostrophanthidin series.[3] In the present instance, we wish to present our experience with digitoxigenin.

In the case of isostrophanthidin, its lactone group has been shown to be the inner ester of the lactol form of a γ-hydroxyaldehyde, and that its formation from strophanthidin involves the

[1] Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, 1926, lxvii, 333.

[2] Jacobs, W. A., Hoffmann, A., and Gustus, E. L., *J. Biol. Chem.*, 1926, lxx, 1.

[3] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, 1927, lxxiv, 811.

disappearance of the double bond of the latter with the production of a new ring. It was at first believed that this isomerization was precipitated by a shift in the double bond induced by enolization of the aldehyde group of strophanthidin under the influence of alkali.* But it has now been found that a number of these aglucones which do not contain free carbonyl groups may be isomerized by alkali.

When digitoxigenin is dissolved in a methyl alcoholic solution of potassium hydroxide, the clear solution becomes, after a short time, a thick mass of lustrous, sparingly soluble crystals of a much higher melting point (271°) and proved to be of neutral character. Analysis showed the substance to possess, like digitoxigenin, the formula $C_{23}H_{34}O_4$. Contrary to digitoxigenin, *isodigitoxigenin* no longer gives the Legal test. On saponification it takes up 1 equivalent of alkali. When the solution of the salt is gently acidified with acetic acid a monobasic acid is obtained—*isodigitoxigeninic acid.* If an excess of mineral acid is employed, relactonization occurs with the reformation of isodigitoxigenin. Isodigitoxigeninic acid is identical with the so called dixgenic acid which was obtained by Kiliani[4] and Cloetta[5] over the sodium salt by the use of aqueous alcoholic alkali. Further study of isodigitoxigenin demonstrated that its relationship to digitoxigenin was similar to that already found between strophanthidin and its iso derivative, and that the transformations already reported in the case of the latter could be repeated exactly here. Contrary to digitoxigenin, isodigitoxigenin resembles isostrophanthidin and cannot be hydrogenated with palladium. The acid, isodigitoxigeninic acid, readily forms a methyl ester with diazomethane. This ester in turn was found to be the ester of a hydroxyaldehydo acid which may exist in either the free aldehydic or lactol form. On the one hand, the methyl ester readily formed a semicarbazone demonstrating the presence of the carbonyl group. On the other hand, if the ester in acetic acid solution was oxidized with chromic acid, it consumed exactly 2 atoms of oxygen with the formation of a neutral substance, $C_{24}H_{34}O_5$, which differed from the original material employed, $C_{24}H_{38}O_5$, by 4 hydrogen

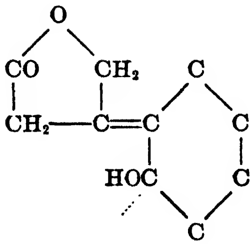
[4] Kiliani, H., *Arch. Pharm.*, 1899, ccxxxvii, 452.

[5] Cloetta, M., *Arch. exp. Path. u. Pharmakol.*, 1920, lxxxviii, 138.

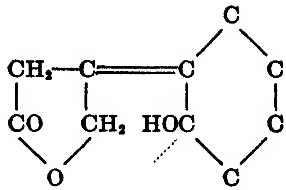
atoms. Although neutral in character, it was found to require 2 equivalents of alkali when saponified, due to the presence of a newly formed lactone group in addition to the original ester group. As seen from the analysis and its ability to form a semicarbazone the lactone ester contains also a carbonyl group so that during its formation the secondary alcoholic group of digitoxigenin is simultaneously oxidized to a ketone. The lactone group of this substance, therefore, could have been produced only by oxidation of the lactol form of a hydroxyaldehyde. After saponification, this lactone ester yielded on reacidification to Congo red a beautifully crystallizing acid which separated slowly as relactonization occurred. This acid, *isodigitoxigonic acid*, exhibited the proper behavior towards alkali. On direct titration it required 1 equivalent for neutralization, and on subsequent heating with an excess of reagent it consumed the additional equivalent required by the lactone group.

The analogy with isostrophanthidin was further confirmed by the behavior of isodigitoxigenin after saponification, that is as isodigitoxigeninic acid, towards hypobromite. This acid was oxidized presumably in the lactol form with the formation of the lactone acid, $C_{23}H_{34}O_5$, or *isodigitoxigenic acid* which differs from the isodigitoxigonic acid described above by the retention of the secondary hydroxyl group.

The results here obtained are exactly analogous to our experience with isostrophanthidin and give direct chemical evidence of the $\Delta^{\beta,\gamma}$ character of the lactone ring of digitoxigenin. Further, this lactone ring is attached by its β-carbon atom to a ring bearing a presumably tertiary hydroxyl group which in turn is most probably γ to the γ-carbon atom of the lactone ring. Alternative possibilities are given by placing the double bond $\Delta^{\beta,\gamma}$ either outside or inside of the lactone ring as shown in the partial formulæ (I) and (IV). In the case of formulæ (I) and (II) the opportunity for *cis-trans* isomerism with reference to the tertiary hydroxyl is shown and the possibility of this structural arrangement playing a rôle in the isomerization of strophanthidin, digitoxigenin, and other similar cardiac aglucones to iso compounds (III) will be discussed in another connection.



I.



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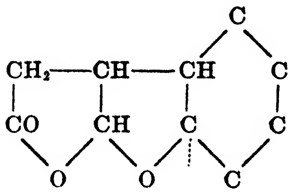
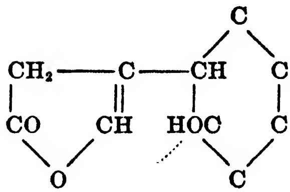


III.



IV.

Parallel studies have been made with gitoxigenin, but in this case a complication has been encountered which will be described in a communication soon to be published. The analytical data obtained with the digitoxigenin derivatives agree best with the figures required for a C_{23} formula, thus confirming the more recent conclusions of Windaus[6] that digitoxigenin is a C_{23} derivative and not a C_{24} compound as presented by Cloetta[7] and then provisionally accepted by Windaus and Freese.[8] Similarly, our analytical data obtained with the gitoxigenin and periplogenin derivatives have shown that these substances are likewise C_{23} derivatives.[9] Further work is in progress on the oxidative degradation of these substances as well as with attempts at their further correlation with strophanthidin. It is of particular interest to determine how far the structural analogies among these substances may be developed.

The digitoxigenin (digitoxin) employed in the present studies

[6] Windaus, A., *Nachr. Ges. Wissensch. Goettingen, Math.- physik. Klasse*, 1926, 170.

[7] Cloetta, M., *Arch. exp. Path. u. Pharmakol.*, 1920, lxxxviii, 133.

[8] Windaus, A., and Freese, C., *Ber. chem. Ges.*, 1925, lviii, 2503.

[9] In a private communication, Professor Windaus has stated that digitaligenin (dianhydrogitoxigenin) is also a C_{23} derivative.

was in part prepared by ourselves directly from digitalis leaves. But we are especially indebted to E. Merck and Company of Darmstadt to whose liberality we owe generous quantities of the insoluble by-product obtained in the manufacture of digitoxin and from which we have prepared both pure digitoxin and gitoxin.

EXPERIMENTAL.

Isodigitoxigenin.—0.2 gm. of digitoxigenin was dissolved in 2 cc. of a solution of potassium hydroxide in dry methyl alcohol (2.4 gm. per 100 cc.) and kept at 20°. After about 20 minutes, crystallization started and the mixture soon became a thick mass of glistening platelets. After 1 hour, the substance was collected and washed with 50 per cent alcohol. It proved to be neutral. The yield was 0.15 gm. Recrystallized from 95 per cent alcohol, it formed narrow platelets or needles which melted at 271°. It is very sparingly soluble in the cold, in methyl and ethyl alcohols, but is appreciably soluble in acetone and chloroform. It is practically insoluble in ether. Contrary to digitoxigenin, it gives no reaction with nitroprusside. In sulfuric acid it dissolves with a slowly developing fluorescent yellow color.

On acidification of the above alkaline mother liquor to Congo red, that portion of the substance which had undergone saponification was relactonized and proved to be identical with the isodigitoxigenin directly obtained.

In an attempt to hydrogenate isodigitoxigenin no hydrogen was absorbed. For analysis the substance was dried at 100° and 15 mm.

3.345 mg. substance: 2.800 mg. H_2O, 9.030 mg. CO_2.

$C_{23}H_{34}O_4$.	Calculated.	C 73.75, H 9.15.
	Found.	" 73.61, " 9.36.

Isodigitoxigeninic Acid (*Dixgenic Acid*).[4]—Isodigitoxigenin was gently heated in 50 per cent alcohol with a slight excess of dilute sodium hydroxide solution until it was dissolved. After cooling, gentle acidification with acetic acid caused the deposition of the acid as broad, flat micro spears, which were completely soluble in dilute carbonate solution. This substance melted with effer-

vescence on rapid heating at about 220°.[10] Upon recrystallization from 50 per cent alcohol, it separated as long, narrow platelets or broad, flat needles which were anhydrous and now melted at 250°.[10]

3.275 mg. substance: 2.785 mg. H_2O, 8.405 mg. CO_2.
$C_{23}H_{36}O_5$. Calculated. C 70.35, H 9.25.
Found. " 69.98, " 9.51.

Isodigitoxigeninic Methyl Ester.—On treatment of a suspension of the acid in dry acetone with diazomethane it slowly dissolved. The residue obtained after removal of the acetone crystallized under methyl alcohol. This was completed by careful dilution, broad six-sided plates being formed. From a small volume of methyl alcohol the ester slowly crystallized as beautifully formed prisms and tables about 0.5 cm. long, which melted at 128°. It is readily soluble in the usual solvents except ligroin. For analysis the ester was again recrystallized by dilution of its alcoholic solution.

3.530 mg. substance: 3.010 mg. H_2O, 9.165 mg. CO_2.
$C_{24}H_{38}O_5$. Calculated. C 70.88, H 9.43.
Found. " 70.80, " 9.54.

Isodigitoxigeninic Methyl Ester Semicarbazone.—0.1 gm. of the ester was dissolved in 10 cc. of alcohol and the solution was treated with 0.1 gm. of semicarbazide hydrochloride and 0.15 gm. of potassium acetate dissolved in 2 cc. of water. After standing 4 days at ordinary temperature, the clear solution was cautiously concentrated in a desiccator. The initial deposition of a gum was followed on rubbing by slow crystallization. Recrystallized by careful dilution of its methyl alcoholic solution, it gradually crystallized as stout prisms and tables which slowly effervesced at 156°. The substance which contained solvent of crystallization was dried for analysis at 100° and 15 mm.

4.260 mg. substance: 3.465 mg. H_2O, 10.030 mg. CO_2.
$C_{25}H_{41}O_5N_3$. Calculated. C 64.73, H 8.92.
Found. " 64.20, " 9.10.

Isodigitoxigonic Methyl Ester.—A solution of 0.7 gm. of isodigitoxigeninic methyl ester in 15 cc. of acetic acid was cooled and

[10] H. Kiliani gives for dixgenic acid the melting point, 220–230° (*Arch. Pharm.*, 1899, ccxxxvii, 452), and M. Cloetta, 243–244° (*Arch. exp. Path. u. Pharmakol.*, 1920, lxxxviii, 138).

treated with 3 cc. of Kiliani chromic acid solution (400 gm. of water, 80 gm. of H_2SO_4, and 53 gm. of CrO_3). Oxidation was prompt and after 5 minutes the mixture which contained a definite excess of the reagent was diluted with water. Glistening leaflets of the neutral lactone ester separated. The yield was 0.6 gm. Upon recrystallization from dilute methyl alcohol, it again separated as leaflets which melted at 190° after preliminary softening and were practically anhydrous. The ester is easily soluble in chloroform, acetone, and alcohol, and is appreciably soluble in ether. In sulfuric acid the solution is practically colorless.

4.660 mg. substance: 3.600 mg. H_2O, 12.175 mg. CO_2.
2.876 " " : 2.230 " " 7.533 " "

$C_{24}H_{34}O_5$.	Calculated.	C 71.60, H 8.52.
	Found (*a*).	" 71.24, " 8.64.
	(*b*).	" 71.42, " 8.67.

For the titration of the substance an as yet unpublished method devised by W. F. Goebel of the Rockefeller Institute was kindly placed at our disposal. For this purpose it was covered with 1 cc. of alcohol and refluxed in an atmosphere of washed N with 3 times the required 0.1 N NaOH solution for 4 hours and then titrated back with 0.1 N acid against phenolphthalein. 14.523 mg. of substance consumed 0.720 cc. of 0.1 N NaOH. Calculated for 2 equivalents, 0.722 cc. 0.1012 gm. of substance consumed 4.96 cc. Calculated for 2 equivalents, 5.03 cc.

Isodigitoxigonic Acid.—On acidification of the above solutions to Congo red, the lactone acid slowly crystallized as leaflets. Recrystallized by careful dilution of the alcoholic solution, it formed thin, anhydrous platelets which melted at 212–213° after slight preliminary sintering.

2.945 mg. substance: 2.195 mg. H_2O, 7.710 mg. CO_2.

$C_{23}H_{32}O_5$.	Calculated.	C 71.08, H 8.31.
	Found.	" 71.39, " 8.34.

11.622 mg. of substance were dissolved in 1 cc. of alcohol and directly titrated with 0.1 N NaOH. Found, 0.297 cc. Calculated for 1 equivalent, 0.299 cc.

The above solution was then treated with 1.4 cc. of 0.1 N NaOH and refluxed for 4 hours as described under the ester. Found, 0.295 cc. Calculated for 1 equivalent, 0.299 cc.

Isodigitoxigonic Methyl Ester Semicarbazone.—0.1 gm. of the ester dissolved in 10 cc. of methyl alcohol was allowed to stand with a solution of 0.1 gm. of semicarbazide hydrochloride and 0.15 gm. of potassium acetate at room temperature for several days. Careful dilution and evaporation gave at first a resin followed by slow crystallization. The collected substance was recrystallized by allowing the diluted methyl alcoholic solution to evaporate. The crystalline residue was collected with 70 per cent methyl alcohol.

The semicarbazone forms practically anhydrous microscopic aggregates of plates and rods which melted with effervescence at 243°.

For analysis, the substance was dried at 100° and 15 mm.

3.460 mg. substance: 2.616 mg. H_2O, 8.286 mg. CO_2.

$C_{25}H_{37}O_5N_3$.	Calculated.	C 65.31,	H 8.12.
	Found.	" 65.30,	" 8.44.

Isodigitoxigenic Acid.—0.15 gm. of isodigitoxigenin was dissolved in 2 cc. of pyridine and treated with 2 cc. of 0.5 N sodium hydroxide solution. The thick pap which formed cleared on gentle warming. After careful neutralization with acetic acid the cooled solution was treated with a solution of 0.15 gm. of bromine in 2.25 cc. of N sodium hydroxide solution. After standing 1 hour at ordinary temperature the reaction product was precipitated with hydrochloric acid and the amorphous acid was collected with water. By careful dilution of its solution in a small volume of alcohol it slowly crystallized as tables and prisms which were collected with cold 50 per cent alcohol. The substance which contained solvent of crystallization melted at 229° after preliminary softening and is readily soluble in alcohol, acetone, and chloroform and practically insoluble in water. In sulfuric acid it gradually forms a yellow solution which on long standing deepens to a deep green.

For analysis the substance was dried at 100° and 20 mm.

3.658 mg. substance: 2.926 mg. H_2O, 9.424 mg. CO_2.

$C_{23}H_{34}O_6$.	Calculated.	C 70.72,	H 8.78.
	Found.	" 70.21,	" 8.95.

11.315 mg. of the lactone acid were treated with 1 cc. of alcohol and directly titrated with 0.1 N NaOH against phenol-

phthalein. Found, 0.298 cc. Calculated for 1 equivalent, 0.291 cc.

The above solution was then treated with 3.5 cc. of 0.1 N NaOH and refluxed for 4 hours, and then titrated back. Found, 0.311 cc. Calculated for 1 equivalent, 0.291 cc.

HYDROGEN ION CONCENTRATION OF FISH MUSCLE.*

By C. C. BENSON.

(*From the Lillian Massey Laboratory of Food Chemistry, University of Toronto, Toronto, and the Atlantic Biological Station, St. Andrews, New Brunswick, Canada.*)

(Received for publication, May 3, 1928.)

In connection with studies of rigor mortis, it seemed worth while to try to find a satisfactory method of determining the hydrogen ion concentration of the muscle.

The quinhydrone method has proved very satisfactory for this purpose, for it allows rapid working and can be so arranged as to cause the minimum of injury to the tissue. Both of these conditions had to be met in these experiments, for Fletcher and Hopkins, (1), working with frog muscle have shown that injury to the tissue stimulates acid production, and Ritchie (2) and Simpson (3) have found for fish that muscle glycogen soon decreases after death, and lactic acid tends to increase very quickly.

Attempts were made to use muscle, frozen with solid carbon dioxide, but without success, and colorimetric determinations with extracts did not give values of sufficient exactness. Muscle, frozen in test-tubes in a brine bath, was used to make cold (3–4°) water extracts. Extracts were made also by dropping the excised muscle into ice water and filtering. The pH of these extracts was taken, quinhydrone being used, but these methods were all troublesome. A few of such determinations are here shown, for the sake of comparison, in Table I, which also shows that results obtained by the procedure described below gave similar results to those obtained with the more elaborate manipulations.

Most of the determinations were obtained by quickly cutting pieces of muscle from the fish immediately after it was stunned, covering a spot of the muscle with the powdered quinhydrone,

* The experimental work was done at the Atlantic Biological Station, St. Andrews, during 1927.

and inserting a stout, bare platinum electrode in order to read the voltage against a calomel electrode with saturated potassium chloride connections. The readings were regularly made with two electrodes and gave the same values; sometimes the bridges,

TABLE I.

Comparison of pH Determined in Different Ways.

Fish No.	Relation to rigor.	pH				
		Quinhydrone method.			Indicator, bromthymol blue.	
		On muscle.	With extract.	With juice.		
Determinations with trawl-caught haddock.						
6	Before.		6.67		6.4	Muscle frozen in brine bath, extracted with water.
	During.		6.67		6.5	
7	Before.		6.49		6.5	
	During.		6.49		6.5	
10	Before.	6.86	6.80			
14	Before.	6.70				Juice squeezed from muscle.
	During.	6.54		6.41		
	After.			6.50		
15	Before.			6 59		
	During.	6.53				
	After.			6.55		
9	Before.		6.28			Extract made by plunging muscle into ice water.
			6.38		6.4	
	During.	6.32	6.34			
	After.	6.43	6.47			
Determinations with minced beef.						
					H_2 electrode with extract.	
	After.	6.27	6.25		6.23	

(of 3 per cent agar and saturated potassium chloride), were changed, and frequently readings were duplicated by changing the places where the bridges made connection, or where the quinhydrone was placed.

In general, values duplicated well but sometimes variations were found as shown in Table II. These variations are to be expected for there are indications that during rigor the musculature of the fish does not change as a whole, but rather that changes are progressive and proceed more rapidly in one place than in another.

The chief difficulty experienced was in inserting the platinum electrode too deeply and so no doubt below the part of the tissue saturated with the quinhydrone.

The matter of temperature correction has also been troublesome for unfortunately the room temperatures during July, 1927, were often high at St. Andrews, where the measurements were made, and the fish muscle was consequently in many cases cooler than was the calomel electrode, for which no means of temperature adjustment had been provided. Biilmann and Krarup (4) have worked out a temperature correction factor for the quinhydrone electrode, and temperature corrections for the calomel electrode are given by Clark (5). Dawson (6) makes use of a formula combining these two factors,

$$\text{pH} = \frac{0.4561 - 0.00014t_1}{0.0541 + 0.002t_1} - \frac{v}{0.0541 + 0.002t} \text{ volts}$$

where v = the reading in volts, t = the temperature of the quinhydrone electrode, and t_1 = the temperature of the calomel electrode. This formula has been used in calculations from the voltage readings. I have also recorded in Table II the millivolts actually read as I have not yet been able to verify fully this correction factor for low temperatures with preparations such as these.

Trawl-Caught Fish.

Determinations were first made with haddock caught by trawl, and a typical experiment of this kind is given in Table II.

Other experiments of a similar kind gave values for pH as shown in Table III. At the bottom of this table are shown the times which the muscle took to reach maximum rigidity. These determinations were made at room temperature except in the case of Fish 12, which was kept in a cold chamber at 12° and was, therefore, slower in becoming hard. The values enclosed in parentheses are those near maximum rigidity.

TABLE II.

Determination of pH of Muscle of Trawl-Caught Haddock.

Fish 13; stunned when caught; maximum rigor (12°) in 5¾ hours.

Time after stunning.	Temperature.		Millivolts.	pH
	t	t_1		
min.	°*C.*	°*C.*		
3	16	21	53	6.85
5	16	21	62	6.69
9	17	21	59	6.75
15	18	21	64	6.66
30	21	21	64	6.69
36	22	22	72	6.51
hrs.				
2⅓	22	22	65	6.63
2½	25	25	60	6.72
3	25	25	56	6.74
4	25	25	67	6.52
5½	26	26	63,70	
24	22	22	72	6.51

TABLE III.

pH Values for Trawl-Caught Haddock.

Time after stunning.	Fish 6.	Fish 7.	Fish 11.	Fish 12.	Fish 14.	Fish 15.	Fish 20.
min.							
3	6.67	6.54	6.64	6.56			6.67
9			6.94	6.75			6.74
hrs.							
½			6.62	6.62	6.70	6.59	6.66
1			6.64	6.51			6.72
2+	(6.67)*	6.49	6.40		(6.54)	(6.53)	(6.68)
3+	6.54	(6.49)	6.40	6.44			6.57
4+			6.40	6.50	6.41		6.70
5½	6.49	6.49	6.42				6.66
20+	5.54			6.51	6.54	6.53	
Maximum rigor at............	3−hrs.	3+hrs.		6 hrs.	3 hrs.	2+hrs.	3 hrs.

* The figures in parentheses denote values near maximum rigidity.

Penned Fish.

The muscle of fish, taken from a pen after 2 weeks or so of quiet, was found to have an alkaline reaction and to change as shown in Table IV.

From these measurements it is evident that the hydrogen ion concentration of muscle is very different for haddock taken from the pen (rested muscle) as compared with that from trawl-caught fish (fatigued muscle). As was to be expected, with penned fish, we find the muscle in alkaline condition; but with trawl-caught fish, the muscle is already near its condition of maximum acidity, due no doubt to the lactic acid produced by struggling.

TABLE IV.

Determinations of pH of Muscle of Haddock from Pen.

Time after stunning.	Fish 10.	Fish 16.	Fish 17.	Fish 21.	Fish 22.	Fish 23.
min.						
3	7.30	7.09	7.16	6.92	7.28	7.30
7	7.31	6.91	6.94	6.80	6.94	7.10
hrs.						
⅓	7.28	7.03	6.88	6.74	6.87	6.96
½	7.26	6.98	6.88	6.80	6.74	6.93
1+	7.13	6.85	6.83	6.85	6.90	6.91
2+	6.88	6.75	6.74	6.74	6.81	7.00
3	6.86		6.94		6.71	
4	6.56	6.74	6.85			
7	6.54					
8	6.56					
9	6.48					
19		6.53				
Maximum rigor at............	16½ hrs.*		16 hrs.*			

* Rigor measured at 12°.

Acidity and Rigor.

The maximum acidity seems, however, to be reached before the muscle showed marked evidence of rigor, and an examination of the variations of pH as compared with the increase and decrease of rigidity, indicates that, while the lower concentrations

of acid at the time of death are associated with the slower progress of rigor mortis, this condition of stiffening does not make itself evident with any definite change of pH value.

These determinations thus fit in with the conception of rigor mortis as a process indirectly related to the accumulation of lactic acid. When one compares the pH values with conditions of rigor mortis, at corresponding times, as found by Panton (7) and others, there is little obvious relationship except that noted by Leim, Macleod, and Simpson (8). This is the fact that the penned fish, which have much higher initial values for pH, pass into rigor much more slowly.

We thus see by actual measurement that the muscle of fish, when they are taken from the pen, is alkaline in reaction. The reaction changes soon to neutrality or to a faintly acid condition and then gradually grows more acid, probably to a pH of about 6.5 or possibly 6.4, though unfortunately it was not realized in time that it was wise to continue experiments long enough to see this, and the low value was only measured in two cases. With these fish, rigor was found to develop very slowly, but reached its maximum some time after the time of greatest acidity.

With trawl-caught fish, the muscle was never alkaline, when the fish were taken from the line. The amount of the acidity varied from a pH just at the neutral point to a value of between 6.5 and 6.6. The acidity increased, but not always regularly, and the minimum values of pH did not correspond with the onset of rigidity or its maximum. The accumulation of acid seems to be initiating some other change, which brings about the stiffening.

pH of Muscle of Hake, Cod, Eel-Pout, and Skate.

In Table V are also given results for a few determinations of pH with other species of fish, all caught by trawl.

These values agree in general with estimations of lactic acid made by Ritchie (9), who noted lower values of lactic acid for cod, than for haddock, and less change in reaction with change in condition of rigidity. He found still lower values for lactic acid in hake.

Further work should be done with skate muscle.

TABLE V.

Determination of pH of Muscle of Fish (Trawl-Caught).

Time after stunning.	Hake 2.	Hake 3.	Cod 1.	Cod 2.	Eel-pout.	Skate 1.	Skate 2.
min.							
6	7.06					7.12	
hrs.							
$\frac{1}{4}$	7.33				6.53	6.82	
$\frac{1}{2}$	7.22			6.97		6.84	
$\frac{3}{4}$	7.28						
1	7.20					6.54	6.56
2	7.24		6.96	6.76	6.54	6.65	6.50
3	7.16		6.90			6.29	
4+	7.10					6.34	
5+	7.22	7.17	7.08		6.44	6.40	
Maximum rigor at............	$1\frac{1}{2}$ hrs.		$2\frac{1}{2}$ hrs.				

Degree of Acidity.

In regard to actual values for pH, these are of the same order of magnitude as those found for cat and for invertebrate muscle by Furusawa and Kerridge (10). Their measurements, taken immediately after death with quickly frozen muscle, varied between pH 6.98 to 7.13, with values, after 20 to 24 hours, of pH 6.10 to 6.52. The pH of 6.10, for tail muscle of *Scyllium canicula*, is lower than any of the values found for haddock muscle, where the lowest value for pH was usually about 6.40. In one case only, Haddock 9 (Table I), the value was as low as 6.28.

SUMMARY.

1. A method is described by which it has been possible to make consecutive measurements of hydrogen ion concentration of muscle over long periods at as frequent intervals as were needed.

2. Determinations of pH of haddock muscle show that fatigued muscle (trawl-caught) is acid at the time of death, may become a little more so, but varies only slightly during the changes of rigidity. The concentration of hydrogen ions is, therefore, not the cause of rigidity.

3. Determinations of pH with rested muscle of haddock (fish taken from a pen) showed an alkaline reaction. This soon changed

to the acid side and then gradually became more acid, approaching the values found for trawl-caught fish.

4. The pH of hake muscle (two tests) was alkaline during 5 hours after death, and never gave an acid reaction.

5. The pH of cod muscle (two tests) was near the neutral point and varied only slightly.

6. The muscle of skate grew slowly more acid in one experiment, of 4½ hours, while the muscle of an eel-pout, which had been out of water for ¾ hour and apparently dead for ¼ hour, showed an acid value for pH during 5 hours of testing.

BIBLIOGRAPHY.

1. Fletcher, W. M., and Hopkins, H. G., *J. Physiol.*, 1907, xxxv, 247.
2. Ritchie, A. D., *Brit. J. Exp. Biol.*, 1927, iv, 327.
3. Simpson, W. W., personal communication.
4. Biilmann, E., and Krarup, I., *J. Chem. Soc.*, 1924, cxxv, 1954.
5. Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd edition, reprinted, 1927, 456.
6. Dawson, L. E., *Sugar*, 1926, xxviii, 212.
7. Panton, J. R., personal communication.
8. Leim, A. H., Macleod, J. J. R., and Simpson, W. W., *Contributions Canad. Biol. and Fisheries*, 1927, iii, 459.
9. Ritchie, A. D., *J. Physiol.*, 1926, lxi, p. iv.
10. Furusawa, K., and Kerridge, P. M. T., *J. Physiol.*, 1927, lxiii, 33; *J. Marine Biol. Assn. U. K.*, 1927, xiv, 657.

THE SOLUBILITY IN THE STOMACH AND DUODENUM OF ALUMINUM COMPOUNDS FOUND IN BAKING POWDER RESIDUES.

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The question has repeatedly been raised as to whether aluminum, when ingested with the food in an insoluble or largely insoluble form such as found in baking powder residues, goes into solution in the stomach. At the time this study was undertaken (1924) the evidence on this point was very limited, although a few *in vitro* and *in vivo* experiments had been conducted. The literature has recently been reviewed by Smith (1).

Experiments were accordingly carried out on human subjects to answer this question. Hospital patients, suffering from disorders which would not influence the results of this study, were given test breakfasts consisting of approximately 100 gm. of biscuit raised with several aluminum baking powders, together with 400 cc. of water. The biscuits were baked for us in the hospital diet kitchen. At the end of 1 hour the gastric contents were siphoned off and analyzed.

The gastric contents were filtered through heavy, folded filter paper, the fluid being returned to the filter if not water-clear. The acidities were titrated with the Töpfer method, while the pH was estimated with the aid of the Myers bicolorimeter, thymol blue being used as the indicator. Aluminum was determined by the Schmidt-Hoagland (2) method, the precipitates being washed with 1 per cent ammonium phosphate as suggested by Balls (3). This method was carefully checked and seemed well suited to the problem at hand. As an additional check on the composition of the aluminum phosphate precipitate, the phosphorus in the precipitate was determined by the Benedict and Theis (4) colori-

metric method and from this the amount of aluminum calculated. Excepting two experiments, the agreement was very close.

TABLE I.

Gastric Analyses on Subjects Receiving Baking Powder Biscuits.

Subject No.	Biscuits ingested.	Baking powder in biscuits ingested.	Gastric contents withdrawn.	pH	Free HCl.	Total acidity.
	gm.	*gm.*	*cc.*		*per cent*	*per cent*
1	100*	0	110	1.3	34	62
2	100	0	88		25	52
3	92	6.07	180	2.2	16	72
4	89	5.87	250	2.4	6	41
5	72	1.15	195	1.9	16	54
6	63	1.00	350	1.4	30	54
7	102	1.63	284	1.4	28	53
8	95	1.52	180	2.4	7	42
9	104	2.84	280	2.2	22	70
10	101	2.75	140	3.0	0	50
11	68	1.85	87	2.1	16	42
12	93	2.53	190	3.0	0	34
13	88	1.24	200			
14	83	1.17	345		0	54
15	89	1.26	214			
16	102	1.44	280			
17	94	1.33	200		18	39
18	108	1.94	175		21	50
19	94	1.78	210		26	65
20	64	1.12	200			

* Subjects 1 and 2 served as controls and received "soda" crackers and yeast bread respectively, Subjects 3 to 8 received biscuits baked with a combination acid calcium phosphate-sodium aluminum sulfate baking powder, while the biscuits given to Subjects 9 to 20 were raised with the aid of two different brands of straight sodium aluminum sulfate baking powder. The biscuits given to Subjects 3 and 4 contained 4 times the amount of baking powder required.

DISCUSSION.

It is believed that the gastric analyses recorded in Table I are fairly representative of what might be found in hospital patients after an Ewald meal containing the amount of bread and water administered. It will be noted in Table II that the gastric contents

removed contained in soluble form from 2 to 20 per cent of the aluminum administered, if the analysis of the filtered gastric contents may be considered as giving the soluble aluminum. In six of the subjects the total aluminum present in the gastric contents removed was also determined. This permitted the calculation of the percentage of soluble (filtrable) aluminum in the

TABLE II.

Soluble Aluminum in Gastric Contents from Baking Powder Residues.

Subject No.	Al in baking powder used.	Al in biscuits ingested.	Total Al in gastric contents.	Soluble Al in gastric contents.	Soluble Al calculated from the P of $AlPO_4$.	Soluble Al in per cent of total Al of gastric contents.	Soluble Al in per cent of Al ingested.
	per cent	*mg.*	*mg.*	*mg.*	*mg.*		
1	0	0		0.12			
2	0	0		0.07			
3	1.52	92.0		14.6	14.5		15.9
4	1.52	89.0		8.1			9.1
5	1.52	17.3		3.3	3.3		19.1
6	1.52	15.1		0.4	0.4		2.7
7	1.52	24.6		2.2	2.3		8.9
8	1.52	22.8		2.1	1.3		9.2
9	2.23	63.4		12.5	10.3		19.7
10	2.23	61.4		1.0	0.8		1.6
11	2.23	41.3		1.7			4.1
12	2.23	56.6		1.5	1.7		2.7
13	3.58	44.7	14.1	1.4		9.9	3.1
14	3.58	42.1	27.7	9.1		32.9	21.6
15	3.58	45.2	23.8	4.7		19.8	10.4
16	3.58	51.8	21.9	1.4		6.4	2.7
17	3.58	47.7	23.2	8.6		37.1	18.0
18	3.58	69.0		3.2			4.6
19	3.58	63.9	20.7	11.1		53.6	17.4
20	3.58	40.3		2.8			7.0

gastric contents. In the six subjects it varied from 6 to 54 per cent and averaged 27 per cent. As will be noted in the tables, there does not appear to be any direct relationship between the gastric acidity and the amount of the soluble aluminum.

If absorption of aluminum takes place, it probably does not occur in the stomach. For this reason observations were made on the content of aluminum in the duodenal contents of four subjects

after they had received 63 mg. of aluminum, on the average, in the form of baking powder biscuits. The duodenal contents were withdrawn with the aid of a duodenal tube inserted from 85 to 100 cm. The soluble aluminum in the four cases was 0.13, 0.55, 0.80, and 0.82, calculated as mg. of Al per 100 cc. In the last two cases the total Al per 100 cc. was 7.47 and 2.74 mg., indicating solubilities of 10.7 and 29.9 per cent respectively. Although our observations on duodenal contents are quite limited, when considered in connection with similar data on the gastric contents, it would seem that they admitted of but one interpretation; namely, that on the average about 25 per cent of the aluminum of baking powder residues found in the stomach and duodenum are present in soluble form. Despite this fact the absorption of aluminum appears to be extremely slight (5, 6).

SUMMARY.

The solubility in the stomach of aluminum compounds found in baking powder residues has been studied in eighteen subjects. With an ingestion of 15 to 90 mg. of aluminum from 0.4 to 14.6 mg. have been found in the gastric contents in soluble form, comprising from 2 to 20 per cent of the amount ingested. In six of the subjects the total aluminum of the gastric contents was also determined. The soluble aluminum, calculated on the basis of the amount actually present in the gastric contents, varied from 6 to 54 per cent, averaging about 25 per cent. The solubility of the aluminum did not seem to bear any direct relation to the gastric acidity. Somewhat similar observations were made on the solubility of aluminum in the duodenal contents of four subjects.

BIBLIOGRAPHY.

1. Smith, E. E., Aluminum compounds in food, New York, 1928, 189.
2. Schmidt, C. L. A., and Hoagland, D. R., *J. Biol. Chem.*, 1912, xi, 387.
3. Balls, A. K., The occurrence of aluminum, and its absorption from food, in dogs, unpublished dissertation, Columbia University, New York, 1917.
4. Benedict, S. R., and Theis, R. C., *J. Biol. Chem.*, 1924, lxi, 63.
5. Myers, V. C., and Mull, J. W., *J. Biol. Chem.*, 1928, lxxviii, 605.
6. Myers, V. C., and Morrison, D. B., *J. Biol. Chem.*, 1928, lxxviii, 615.

THE ESTIMATION OF ALUMINUM IN ANIMAL TISSUES.*

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The question of the absorption of aluminum from the alimentary tract and its presence in various tissues, when diets both with and without aluminum were fed, has been a topic of discussion for many years. No definite attempts were made to answer these questions, however, until about 1911 when several studies were reported from Gies' laboratory. Steel (1), Kahn (2), and later Balls (3) attempted to ascertain the presence of aluminum in normal tissues and studied the influence of ingested aluminum. Both Steel and Kahn employed the method of the Association of Official Agricultural Chemists, slightly modified (4), while Balls used the later Schmidt and Hoagland (5) method. This method was also employed by the Referee Board (6) to estimate the aluminum content of the blood of subjects receiving aluminum from baking powder residues. The Schmidt-Hoagland method, in particular, is a most excellent one when the quantities of aluminum are sufficient for accurate gravimetric work, as for

* The data presented in this and the two following papers are taken from the dissertations submitted by James W. Mull and Dempsie B. Morrison to the Graduate College of the State University of Iowa, July, 1927, and June, 1928, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

A report of the work given in this and the three following papers was presented before the American Society of Biological Chemists, at Ann Arbor, Michigan, April 12, 1928. A preliminary report of the method was presented before the Iowa Branch of the Society for Experimental Biology and Medicine, February 2, 1927 (Mull, J. W., Morrison, D. B., and Myers, V. C., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 476).

example in the feces of aluminum-fed subjects (7), but the amounts of aluminum present in animal tissues are so small, and the inorganic constituents so large, that the data which have been reported with this and other gravimetric methods are of questionable value.

Since our work was completed a book by Smith (8) on aluminum compounds in foods has appeared, in which figures for a number of unpublished analyses are given. Some of these harmonize with our findings very well, notably those of Killian (9) and Gray (10). The former employed the Schmidt-Hoagland method, while the latter used the Atack (11) alizarin method, slightly modified.

In the present study a delicate colorimetric method has been used, which, although less accurate than gravimetric methods where large amounts are to be determined, is unquestionably better suited to the estimation of minute amounts of aluminum in animal tissues. Three very delicate color reagents for aluminum have been described: alizarin by Atack (11) in 1915, the ammonium salt of aurin tricarboxylic acid by Hammett and Sottery (12) in 1925, and morin by Schantl (13) in 1924, the last named having thus far been used only in a qualitative way. The method of Hammett and Sottery, with certain modifications, has proved most suitable, the aurin being less affected by slight changes in the acid concentration and showing less tendency to salt out than the alizarin used by Atack.

The amount of aluminum present in biological tissues is so small, in comparison with the other inorganic constituents, especially iron, that it was found necessary to remove these before making the colorimetric tests. The ordinary methods of separation proved quite inadequate for this as the small amounts of iron left are sufficient to give a color with the dye so similar to that of aluminum as to invalidate the results. It was later found that, although iron must be completely removed before the development of the color reaction, it must be present at the time of the first precipitation of the aluminum, otherwise the aluminum is not completely precipitated. The following method of separation and subsequent estimation has been worked out and found to be entirely successful for the purpose.

Method.

Digestion of Tissues.—The tissues to be analyzed are digested until all organic matter has been destroyed in Pyrex or silica flasks of a capacity convenient for the amount of tissue employed. 50 gm. of tissue may be handled in 300 cc. flasks, but this amount requires careful manipulation. Blanks run with both Pyrex and silica flasks have shown that either are suitable for the work. The digestions are made with a mixture of concentrated sulfuric acid and 60 per cent perchloric acid, roughly in the proportion of 1 to 1.25, with an amount that will keep the material semiliquid at all times. A few silica chips are an aid in overcoming the bumping which is apt to occur as the water vapor is driven off, just before charring, but which is usually not severe. The flasks are heated with a very low flame, so that the contents barely boil, the flame being removed if the action becomes intense. This method prevents a rather violent exothermic reaction which otherwise takes place, accompanied by a tremendous evolution of fumes. During the course of the digestion the material chars, becomes thick, then begins to clear up, the final product being a water-clear liquid, which generally contains a fine white deposit. This liquid should be concentrated to a small volume, 5 cc. or less. A suction tube, introduced part way down the neck of the flask, markedly accelerates this operation by removing the heavy acid fumes which are otherwise driven off with difficulty.

Removal of Iron and Salts.—The digested product, including the precipitate which is loosened with a glass rod and partially dissolved in concentrated HCl, is transferred to a volumetric flask and made up to volume. If a very little aluminum is expected the whole of the digestion is used, otherwise an aliquot is taken. The amount used is pipetted into a 50 cc. conical centrifuge tube and 1 mg. of ferric iron added, if the amount of iron in the tissue is very small. The contents are then diluted to about 15 cc. with distilled water, neutralized with ammonium hydroxide, 1 drop of very dilute methyl red being used as indicator, and 1 cc. of a saturated solution of ammonium acetate added.

The tubes are then placed in a boiling water bath for 10 minutes or more, until the precipitate has begun to settle, centrifuged, and the liquid decanted off and discarded. This removes all the

soluble salts. The precipitate is dissolved as completely as possible in 1 cc. of 6 N HCl, with warming when necessary, and the solution diluted to 15 cc. To this solution, cooled to room temperature, is added 1.25 cc. of glacial acetic acid and 5 cc. of 6 N NaOH prepared from metallic sodium (special aluminum-free). With occasional agitation the tubes are allowed to stand an hour, to insure complete precipitation of the iron and separation from aluminum, then, after washing down the sides with distilled H_2O, they are centrifuged, and the liquid decanted into 50 cc. Nessler tubes.

Development of the Color.—To the solution in the Nessler tubes are added 1.1 cc. of 6 N HCl and 0.75 cc. of glacial acetic acid and the volume made up to between 35 and 40 cc. with distilled water. A 0.1 per cent solution of the ammonium salt of aurin tricarboxylic acid[1] is then added in the proportion of 1 cc. for each 0.01 mg. of aluminum, the tubes inverted, and thoroughly shaken. 10 minutes are permitted for the formation of the lake in this acid solution, after which it is neutralized with an ammonium hydroxide-carbonate mixture. This is made by dissolving 180 gm. of $(NH_4)_2CO_3$ in 420 cc. of concentrated NH_4OH and 1700 cc. of water. 5 cc. of this mixture should bring the pH of the determination to between 7.0 and 7.3, but the amount may be varied if necessary. After the mixture is inverted and shaken thoroughly, the colors are compared against standards, the full length of the Nessler tubes being used. It is advisable to shake and invert all the tubes a fixed number of times in order that the evolution of CO_2 may be as nearly uniform as possible. The bubbles formed by the escape of this gas make the use of a colorimeter rather unsatisfactory. At first we employed a 100 mm. Duboscq colorimeter but later, owing to the bubbles and lightness of the colors, we abandoned this in favor of the 200 mm. Nessler tubes.

Fresh standards[2] are prepared for each determination. These

[1] Prepared by dissolving 1 gm. of aurin tricarboxylic acid (Eastman) in about 200 cc. of water and 2.5 cc. of concentrated NH_4OH and making up to 1 liter. The solution should not be left exposed to the light.

[2] A stock aluminum standard may be prepared by dissolving 3.5018 gm. of potassium alum in distilled water and diluting to 1 liter. 50 cc. of the stock solution make a liter of a standard, containing 0.01 mg. of Al in 1 cc. Our potassium alum was five times recrystallized and carefully dried over $CaCl_2$ under reduced pressure.

differ by 0.005 mg. of aluminum and cover the desired range, usually from 0.005 mg. to 0.035 mg., although from 0.00 to 0.06 mg. may be used. They are made by taking the necessary amounts of the standard aluminum solution, prepared from recrystallized potassium aluminum sulfate, and adding the same reagents used in the unknowns; *i.e.*, 5 cc. of NaOH, 2.1 cc. of 6 N HCl, 2 cc. of glacial acetic acid, the correct amount of dye, and ammonium hydroxide-carbonate mixture. The comparisons are made as soon as the bubbles pass off. The pH of the solutions is then taken colorimetrically, phenol red being used, and all tubes falling outside the range of 7.0 to 7.4 are discarded.

DISCUSSION.

It is of the greatest importance in using the method described to have pure reagents. The digesting acids must be free from aluminum and all materials used in the later steps must be free from iron as well. Satisfactory sodium hydroxide is particularly difficult to obtain. Only that prepared from metallic sodium can be used, and some brands of this contain enough iron or aluminum to make them unsuitable. The aurin tricarboxylic acid must be carefully tested, as some of the preparations on the market do not give good color lakes. Ammonium hydroxide which has stood for some time in glass may give a test for aluminum and in this case should be redistilled before being used.

The amount of aluminum present in the digestion as used is usually so small that it cannot, of itself, be precipitated by ammonium hydroxide. In the presence of iron, however, the precipitation is quantitative. It is therefore necessary to add iron to the aliquots taken, unless it is already present, even though it must be removed later to prevent interference with the aurin aluminum color lake. The effectiveness of the precipitation is also increased by the presence of the acetate ion with both the NH_4OH and NaOH. In the latter case satisfactory removal of the iron is entirely dependent on the amount of acetic acid used. Increasing the proportion causes loss of aluminum, while a decrease permits iron to go through.

In applying this method a number of limitations must be taken into consideration. First it is designed for very small amounts of aluminum. Determinations are not accurately made with

amounts of 0.1 mg. of aluminum or more and we personally find the range below 0.06 mg. preferable. We do not, therefore, recommend this method when the aluminum content exceeds 1.0 mg. per 100 gm. of tissue.

The nature and amount of the other inorganic constituents are also limiting factors. If the precipitate resulting from the neutralization with ammonium hydroxide is too great to be dissolved in 1 cc. of 6 N hydrochloric acid, even after dilution to 15 cc., the determination is impossible with the directions given. Similarly, if the iron precipitated by the sodium hydroxide forms a bulk of over 3 cc., after it has been centrifuged, it will hold aluminum and produce inaccurate results.

The most probable source of error in the method is apparently that of contaminations introduced during the digestion. We have checked this in several ways. The acids have been tested separately and have been found aluminum-free. Blanks have been run in which no tissue was used, to which not only iron but, at times, magnesium and calcium phosphate were added. These tests seem to check, and show a total of not more than 0.01 mg. of aluminum present in the entire digestion mixture. Since this amount represents a contamination present in almost all material, we have considered the digestion method satisfactory. The failure to find very little, if any, aluminum in certain tissues, notably blood, seems to strengthen our position.

The possible introduction of aluminum with the reagents subsequently used is of no consequence, since the same amounts of these reagents are also used in the standards. There remains the possibility of loss of aluminum, or of the presence of some other element that will form the color lake. The former possibility has been checked by adding aluminum to samples and carrying them through the process; the latter by making an analysis, adding aluminum to another aliquot, and finding the calculated total on the basis of the first analysis. It is improbable that any other element in the tissues would escape elimination and give a color addable to that of aluminum. Additional evidence is supplied by spectrograms of the digested material (kindly furnished by Dr. O. S. Rask) which show the lines characteristic of aluminum.

In Table I are presented experiments which show that iron, calcium, magnesium, and phosphate are removed with sufficient

TABLE I.

Checks on Purity of Reagents and Removal of Other Elements.

Materials added.							Al found.
85 per cent H_3PO_4.	Fe	Concentrated H_2SO_4.	Mg	$Ca_3(PO_4)_2$	$CaCl_2$	Al	
cc.	*mg.*	*cc.*	*mg.*			*mg.*	*mg.*
0.25	0.3	1				0.01	0.01
0.25	0.5	1				0.01	0.01
0.25	0.6	1				0.01	0.01
0.25	1.0	1				0.01	0.01
	1.0	1					0.001
	1.0	1				0.005	0.005
Present.	0.5				Present.		0.000
	0.3					0.01	0.01
	0.3				Present.	0.02	0.02
Present.	0.3				"	0.02	0.02
	1.0	1		Present.		0.015	0.015
	1.0	1	10			0.005	0.005

TABLE II.

Checks on Digestion and Recovery of Added Aluminum.

Materials present.					Al found.
H_2SO_4	$HClO_4$	Tissue.		Al	
cc.	*cc.*		*gm.*	*mg.*	*mg.*
15	20	*			0.01
15	20	†			0.01
15	20	Liver.	50.5		0.03
15	20	"	52	0.05	0.092
20	25	Kidney.	49.5		0.023
20	25	"	47.0	0.05	0.073
20	25	Heart.	50.8		0.120
20	25	"	51.2	0.05	0.160
20	25	Liver.	40.0		0.080
20	25	"	40.0	0.10	0.176
20	25	Heart.	40.0		0.036
20	25	"	40.0	0.07	0.100
20	25	Liver.	40.0		0.066
20	25	"	40.0	0.07	0.130
3	5	Blood.	5.0		0.005
3	5	"	5.0	0.01	0.013
3	5	"	5.0	0.02	0.024
3	5	"	5.0	0.03	0.033

* 5 mg. of Fe added.

† 5 mg. of Fe, 10 mg. of Mg, and 25 mg. of $Ca_3(PO_4)_2$ added.

TABLE III.

Checks on Final Colorimetric Readings.

Aliquot of tissue digestion.	Al added.	Al found.	Al in 5 cc. of digestion mixture calculated from determinations.
cc.	*mg.*	*mg.*	*mg.*
10	0.01	0.012	0.001
15		0.007	0.002
5		0.01	0.1
5	0.01	0.022	0.012
5	0.01	0.022	0.012
10		0.005	0.0025
15		0.007	0.0023
10	0.005	0.009	0.002
10	0.005	0.011	0.003
10		0.008	0.004
10		0.007	0.0035
5	0.01	0.012	0.002
5		0.002	0.002
5	0.005	0.007	0.002
10		0.007	0.0035
10		0.007	0.0035
15		0.008	0.0026
5	0.01	0.012	0.002
5	0.005	0.014	0.009
10		0.019	0.0095
5		0.013	0.013
5	0.005	0.018	0.013
4		0.011	0.013
5		0.012	0.012
5		0.012	0.012
5	0.01	0.022	0.012
5	0.005	0.017	0.012
10		0.025	0.0125
10		0.020	0.010

completeness to prevent interference with the final colorimetric reading. Excessive amounts of calcium give too great a precipitate to be dissolved in the 6 N HCl, as mentioned above, while phosphoric acid decolorizes the aurin. The amounts of these elements found in the tissues, however, have not been large enough to cause this difficulty. The table also indicates the freedom from aluminum of the sulfuric acid and iron solution used.

Table II gives some checks on the method of digestion, showing the small amount of aluminum found after the reagents have been carried through the process, and the recovery of aluminum added before digestion. In these cases the same samples of tissue were employed and the two digestions carried on simultaneously.

Table III includes a few of the checks made on the final readings by adding aluminum to aliquots of each digestion. The recovery of the calculated total shows that there is no loss of aluminum during the process, and that the color formed by the unknown is addable to that of aluminum. Use of different amounts of the digestion material is a further proof that the determinations are quantitative.

SUMMARY.

A colorimetric method of estimating the minute amounts of aluminum, which may be found in the various body tissues and fluids, is described. The essential features of the method are: (1) the digestion of the tissues with a sulfuric-perchloric acid mixture, (2) the precipitation of the aluminum along with a very small amount of iron and the subsequent complete separation from the iron, and (3) the development of a color reaction upon the aluminum with the ammonium salt of aurin tricarboxylic acid. The method is adequate for estimating the aluminum present in tissues under various conditions; *i.e.*, amounts varying from less than 0.01 to 0.5 mg. per 100 gm. of tissue. It is not suited to amounts of aluminum exceeding 1.0 mg. per 100 gm. of tissue. Owing to the delicacy of the method only reagents of the very highest purity can be used, but with suitable reagents and careful manipulation the error of the method should not exceed 10 per cent.

BIBLIOGRAPHY.

1. Steel, M., *Am. J. Physiol.*, 1911, xxviii, 94.
2. Kahn, M., *Biochem. Bull.*, 1911–12, i, 235.
3. Balls, A. K., *Biochem. Bull.*, 1916, v, 195, and unpublished dissertation, Columbia University, New York, 1917.
4. Steel, M., *Biochem. Bull.*, 1916, v, 173.
5. Schmidt, C. L. A., and Hoagland, D. R., *J. Biol. Chem.*, 1912, xi, 387.
6. Referee Board of Consulting Scientific Experts, *U. S. Dept. Agric., Bull. 103*, 1914.
7. Schmidt, C. L. A., and Hoagland, D. R., *Univ. Calif. Pub. Path.*, 1919, ii, 215.
8. Smith, E. E., Aluminum compounds in food, New York, 1928.
9. Killian, J. A., unpublished analyses cited by Smith (8).
10. Gray, P. B., cited by Smith (8).
11. Atack, F. W., *J. Soc. Chem. Ind.*, 1915, xxxiv, 936.
12. Hammett, L. P., and Sottery, C. T., *J. Am. Chem. Soc.*, 1925, xlvii, 142.
13. Schantl, E., *Mikrochem.*, 1924, ii, 174.

THE INFLUENCE OF THE ADMINISTRATION OF ALUMINUM UPON THE ALUMINUM CONTENT OF THE TISSUES, AND UPON THE GROWTH AND REPRODUCTION OF RATS.

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Experiments have been carried out on rats for the purpose of studying the influence of aluminum administration upon the content of aluminum in the tissues, and upon growth and reproduction.

EXPERIMENTAL.

The animals used, a mixture of pied and albino rats, were fed the same stock diet.[1] No effort was made to select a diet free from aluminum, but rather an adequate normal diet. The controls received only the stock diet while the experimental animals received in addition 2 mg. of aluminum per rat daily in a small biscuit made of flour and water, sweetened with saccharin, and dried in an oven until quite hard. This amount of aluminum, in the form of potassium aluminum sulfate, represents an average of 8 mg. of aluminum per kilo of body weight, and is 2 to 3 times that which the Referee Board (1) considered a large amount. All aluminum diet rats were kept on this diet for at least 100 days

[1] The diet consisted of: cracked yellow corn; soy beans, cooked 30 minutes under 20 pounds steam pressure; NaCl and $Ca_3(PO_4)_2$; together with about 25 cc. of pasteurized milk, 0.4 gm. of raw liver, 0.2 gm. of powdered Yeast Foam Tablets, and 3 drops of cod liver oil per rat per day. The salts were added to the cooked beans, 1 tablespoonful of each to a quart of beans. Roughly 3 parts of corn to 1 of beans were fed in the amounts the animals would take each day. No exact measures were made of the milk, liver, or yeast, but amounts approximating the quantitites stated were fed to each animal. The cod liver oil was added to the milk. Fresh water was supplied daily.

before being sacrificed for analysis. After the first generation the young were raised, weaned, and at once put on the experimental diet, so that they received the aluminum-containing biscuits from the time of weaning. The animals were killed for analysis by bleeding under ether anesthesia.

TABLE I.

Control Rats.

Generation.	Tissue.	Animals.	Tissue digested.	Analyses.	Al found.	Average Al content.
			gm.		*mg. per 100 gm.*	*mg. per 100 gm.*
2nd	Liver.	6	44.5	3	0.21	
2nd	"	6	34.5	2	0.07	
3rd	"	3	20.0	3	0.23	
3rd	"	5	43.0	2	0.14	
4th	"	8	60.5	2	0.06	0.14
2nd	Heart and tongue.	12	18.0	2	0.29	
3rd	" " "	5	8.0	1	0.11	
4th	" " "	8	12.0	1	0.09	0.16
4th	Thyroid.	8	7.0	1	0.18	0.18
2nd	Adrenal and kidney.	12	17.0	3	0.15	0.15
2nd	Spleen.	12	9.0	3	0.29	0.29
2nd	Lung.	12	12.0	3	0.00	
4th	"	8	12.0	1	0.13	0.06
2nd	Brain.	12	15.0	3	0.00	
4th	"	8	13.0	1	0.09	0.05
1st	Fetal young.	10	30.0	3	0.33	0.33
2nd	Young rat.	1	9.0	3	0.14	
2nd	" "	4	17.5	2	0.23	
2nd	" "	5	17.0	3	0.33	
2nd	" "	1	12.0	2	0.10	0.20
3rd	Blood.	3	21.0	2	0.05	
3rd	"	5	35.5	2	0.07	
4th	"	8	65.0		0.06	0.06

Aluminum Content of Tissues of Rats.

In making the analyses it was found necessary to combine the same tissues from animals in the same group in order to have sufficient material for satisfactory analytical work. The estimations of the aluminum were made by the method described in the preceding paper (2). The results obtained on the rats on the control

TABLE II.

Rats on Aluminum Diet.

Generation.	Tissue.	Animals.	Tissue digested.	Analyses.	Al found.	Average Al content.
			gm.		*mg. per 100 gm.*	*mg. per 100 gm.*
1st	Liver.	4	33.0	2	0.19	
1st	"	3	29.5	2	0.30	
2nd	"	4	25.0	2	0.23	
2nd	"	3	22.0	3	0.23	
3rd	"	12	101.0	4	0.11	
4th	"	8	63.5	2	0.12	0.19
1st	Heart.	7	7.5	1	0.24	
2nd	"	7	6.0	2	0.23	
3rd	"	12	11.0	1	0.12	0.20
2nd	Tongue.	7	5.5	2	0.00	
3rd	"	12	9.0	1	0.10	0.05
4th	Heart and tongue.	8	13.0	1	0.12	0.12
3rd	Thyroid.	12	11.0	1	0.21	
4th	"	8	7.5	1	0.16	0.19
1st	Kidney and adrenal.	7	13.0	1	0.00	
2nd	" " "	7	11.0	3	0.00	
3rd	" " "	12	21.5	1	0.10	0.03
1st	Spleen.	7	7.5	1	0.10	
2nd	"	7	6.0	2	0.00	
3rd	"	12	10.0	1	0.05	0.05
1st	Lung.	7	10.0	1	0.00	
2nd	"	7	10.0	2	0.00	
4th	"	8	12.5	1	0.14	0.05
1st	Brain.	7	11.5	1	0.39	
2nd	"	7	9.0	2	0.00	
3rd	"	12	21.0	1	0.15	0.18
3rd	Fetal young.	6	33.5	3	0.00	
4th	" "		23.0	1	0.09	0.05
2nd	Young rat.	1	13.0	1	0.15	
3rd	" "	5	23.0	3	0.06	
3rd	" "	5	24.0	1	0.12	
3rd	" "	6	22.0	2	0.14	
3rd	Blood.	12	106.0	3	0.00	
4th	"	8	67.5	3	0.01	0.00
1st	Testes.	4	11.0	1	0.36	
2nd	"	3	8.5	3	0.00	
3rd	"	5	13.0	1	0.12	
4th	"	4	11.0	1	0.09	0.14

diet are presented in Table I. There is considerable variation between the different analyses made, but it is believed that the averages furnish a fairly reliable index of the amounts of aluminum to be found in the different tissues. Similar determinations on the aluminum diet rats are given in Table II. There are no outstanding differences. In many cases the variations within the group are greater than those between the groups. However, the livers of the diet animals do run slightly higher in the maximum and minimum figures as well as in the average. The same is true of the brain tissue. Concerning other tissues no conclusions can be drawn.

TABLE III.

Rats on Aluminum-Free Diet.

Group No.	Tissue.	Animals.	Tissue digested.	Analyses.	Al found.	Average Al content.
			gm.		*mg. per 100 gm.*	*mg. per 100 gm.*
1	Liver.	3	23.5	2	0.13	
2	"	5	51.5	2	0.03	0.08
2	Heart and tongue.	5	10.5	1	0.06	0.06
2	Kidney and spleen.	5	16.0	1	0.07	0.07
2	Lung.	5	8.0	1	0.09	0.09
2	Brain.	5	9.5	1	0.20	0.20
2	Blood.	5	50.0	3	0.02	0.02

Since our experiments were completed a paper has appeared by Flinn and Inouye (3) in which they discuss the distribution of aluminum, copper, nickel, tin, and zinc in the various organs of the body when these metals are given in the food. Rats were used as the experimental animal and received 2 mg. of the metal a day. The analytical figures which they have given appear to be somewhat higher than those which we have found. It is difficult to compare their findings with our data, however, in view of the fact that in most cases their figures are given in mg. per rat instead of mg. per 100 gm. of tissue. They make no mention of the analytical methods which they employed.

A few rats, raised by Dr. Amy L. Daniels of the Child Welfare Research Station, on a diet as free from aluminum as possible, were analyzed. The results, given in Table III, show small amounts

of aluminum still present, indicating that it is either retained in the body or that it is very difficult to exclude from the food. The amount found in the brain in this case seems out of harmony with the other tissues and therefore may be open to question.

In order to study the effects and fate of aluminum when present in the body, several rats were injected intraperitoneally with 4 mg. of aluminum in the form of the potassium aluminum sulfate. No ill effects were observed, the animals continuing to gain normally and function as before. Analyses of the tissues and excretions, given in Table IV, show in particular a marked increase in the aluminum content of the liver, with elimination by the intestines

TABLE IV.

Rats Receiving Aluminum by Intraperitoneal Injection.

Material.	Amount digested.	Al found.	Al in sample used.	Days after injection of 4 mg. of Al.
	gm.	*mg. per 100 gm.*	*mg.*	
1 liver.	11.0	9.20	1.01	2
1 "	10.5	7.25	0.76	5
Blood and organs.	31.5	0.29	0.09	2
" " "	32.0	0.26	0.08	4
Feces.	7.5	9.73	0.73	9 day output.
"	2.0	10.00	0.20	3 day output, 2nd to 5th.
	cc.			
Urine.	31	0.32	0.10	3 day output, 2nd to 5th.

and probably to a small extent by the kidneys. It was not possible to rule out completely the contamination of the urine with the feces. These findings indicate that aluminum can be transported in the body, apparently by the blood stream. The large amount of aluminum in the liver would suggest its excretion into the intestines in the bile.

Influence of Aluminum upon Growth and Reproduction.

During the course of this work four generations of animals were raised, and the fifth weaned. Growth in both the control and

aluminum-fed groups was above Donaldson's (4) normal for male and female. In Fig. 1 are given the composite curves entirely typical of the second, third, and fourth generations. Since the

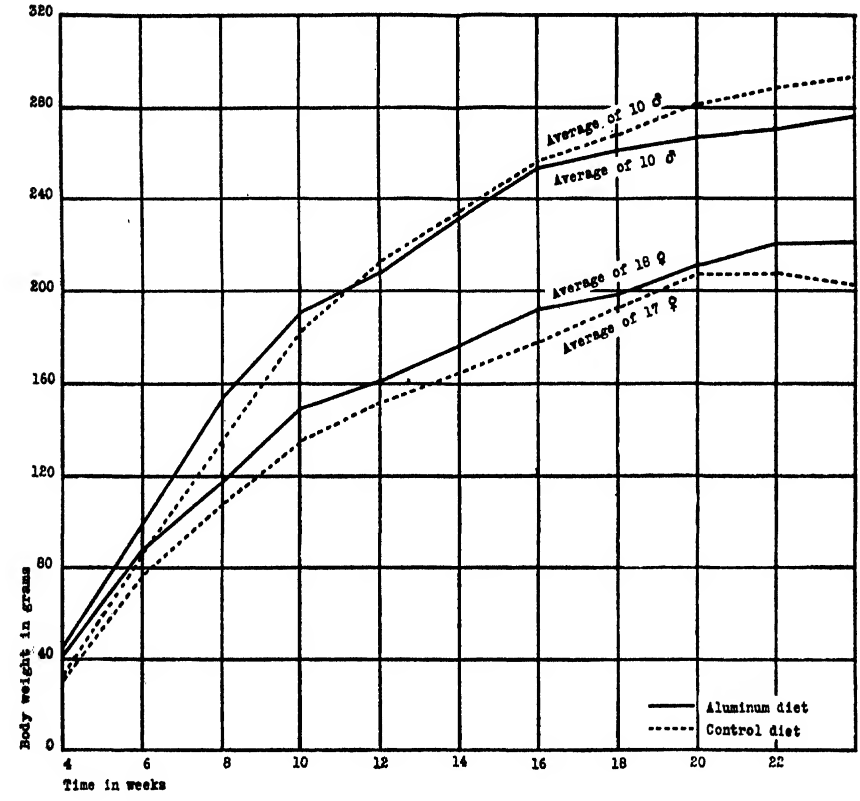

FIG. 1. The composite growth curve includes all the adult animals of the second, third, and fourth generations, distributed as follows: control group, second generation 4 males, 8 females; third generation 4 males, 6 females; fourth generation 2 males, 3 females; experimental group, second generation 3 males, 9 females; third generation 4 males, 6 females; fourth generation 3 males, 3 females. In addition to the stock diet each experimental rat received 2 mg. of aluminum per day.

first generation is not included, all the experimental animals represented were subject to the effect of the aluminum diet from the time of their conception. The only manifestation brought out by the curves, however, is a slightly more rapid initial growth

with the aluminum-fed stock. It is of interest in this connection that as early as 1913 Osborne and Mendel (5) reported significant traces of aluminum, fluorine, and manganese in milk and found that when traces of these elements were added to their inorganic salt mixture better growth was obtained. Daniels and Hutton

TABLE V.

Influence upon Reproduction of Addition of Aluminum to Diet.

Diet.	No. of females.	Average age.	Young born.	Average litter.	Per cent raised.
First litters.					
		days			
Control..	16	106	86	5.4	66
Aluminum	23	104	150	6.5	40
Second litters.					
Control	3		25	8.3	64
Aluminum	6		56	9.3	78

TABLE VI.

Influence of Addition of Aluminum to Diet upon Excretion of Feces and Urine.

Diet.	No. of rats.	Average weight.	Days of collection.	Feces.		Urine.	
				Amount collected.	Daily average.	Amount collected.	Daily average.
		gm.		*gm.*	*gm.*	*cc.*	*cc.*
Aluminum	4	272	7	35.5	1.27		
Control	4	281	7	34.5	1.23		
"	4	212	2	8.5	1.06		
Aluminum	1	340	2	4.0	2.00		
Control	4	212	2			37	4.6
Aluminum	4	234	2			43	5.4
Control	4	226	2			43	5.4
Aluminum	4	256	2			34	4.3

(6) have shown that the addition to milk of these elements present in milk in low concentration, *viz.* aluminum, fluorine, and manganese, together with silicon, seem to be essential to satisfactory reproduction, an observation which has been confirmed by Mitchell and Schmidt (7). In view of our observations on the influence of

aluminum on growth (Fig. 1), the following statement of Hubbell and Mendel (8) regarding zinc is interesting; *viz.*, "The slightly favorable effect of adding 0.02 mg. was more evident with the females than with the males."

Comparisons on reproduction are made in Table V. Free intercourse was permitted until after the birth of the first litters, which came quite early. These were of normal size, the number still-born was almost negligible, and the percentage raised fairly good for the first litters.

Marked improvement is shown in the second litters, though due to lack of space fewer of these could be permitted. It is evident, however, that the diet was not the cause of the low percentage reared in the first litters, since such marked improvement was made during its continuation. One female, of the second generation, successfully raised eight young out of ten after 326 days on the aluminum diet, indicating no cumulative effect.

A comparison of the urinary and fecal excretions is shown in Table VI. The daily averages are almost the same when the collections were made over the same periods of time from males of about the same size and age. The stools were always formed, and no evidence of diarrhea was noted at any time. Similarly the urine was normal, no proteinuria being observed.

No abnormalities were noted grossly at autopsy and all animals appeared healthy. The animals having received the intraperitoneal injections showed no evidence of it. That their bodily functions were unimpaired is shown by the fact that they not only continued to grow at a normal rate, but were bred and bore young as before.

SUMMARY.

The aluminum content of the tissues of rats has been determined on four groups of animals: (1) on a control diet, (2) on a diet with a high aluminum content, (3) on an aluminum-free diet, and (4) following the intraperitoneal administration of aluminum.

Minute traces of aluminum are present in the tissues normally and these show only a slight increase on a diet containing considerable aluminum. Aluminum persists in the tissues even on an aluminum-free diet.

Following intraperitoneal administration, aluminum is found

in increased amounts throughout the body, the largest amounts apparently being in the liver. It appears to be excreted chiefly by way of the intestine.

For the liver the average findings of aluminum per 100 gm. of tissue were: (1) control diet 0.14 mg., (2) aluminum diet 0.18 mg., (3) aluminum-free diet 0.08 mg., and (4) aluminum administered intraperitoneally 8.22 mg.

Observations have been made covering four generations of rats receïving 2 mg. of aluminum in the form of potassium aluminum sulfate per rat daily in addition to the stock diet. The growth curves of these animals compare well with those of the controls, the only difference being that the aluminum-fed rats show a slightly greater initial growth. So far as could be ascertained the addition of aluminum to the diet was without other influence.

Addendum.—Since the present study was completed and submitted for publication, a paper has appeared by McCollum, Rask, and Becker (9) on a study of the possible rôle of aluminum compounds in animal and plant physiology, in which the rat was employed as the experimental animal. They noted no deleterious action of aluminum compounds upon growth, reproduction, or general well being of the animals. They employed the spectrographic method to estimate aluminum, but obtained essentially negative results on the tissues of both the control and aluminum diet rats.

BIBLIOGRAPHY.

1. Referee Board of Consulting Scientific Experts, *U. S. Dept. Agric., Bull. 103*, 1914.
2. Myers, V. C., Mull, J. W., and Morrison, D. B., *J. Biol. Chem.*, 1928, lxxviii, 595.
3. Flinn, F. B., and Inouye, J. M., *J. Am. Med. Assn.*, 1928, xc, 1010.
4. Donaldson, H. H., The rat, Philadelphia, 2nd edition, 1924.
5. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, xv, 311.
6. Daniels, A. L., and Hutton, M. K., *J. Biol. Chem.*, 1925, lxiii, 143.
7. Mitchell, H. S., and Schmidt, L., *J. Biol. Chem.*, 1926, lxx, 471.
8. Hubbell, R. B., and Mendel, L. B., *J. Biol. Chem.*, 1927, lxxv, 567.
9. McCollum, E. V., Rask, O. S., and Becker, J. E., *J. Biol. Chem.*, 1928, lxxvii, 753.

THE INFLUENCE OF THE ADMINISTRATION OF ALUMINUM UPON THE ALUMINUM CONTENT OF THE TISSUES OF THE DOG.

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In the study of the absorption of aluminum from the alimentary tract reported in the preceding paper (1) the rat was employed as the experimental animal. Owing to its small size and to the minute amounts of aluminum found to be present in animal tissues, the rat did not prove to be an ideal animal for this work, despite the very great delicacy of the analytical method used. For this reason a second study was undertaken on dogs.

The dog was the experimental animal employed in the experiments conducted by Steel (2), Kahn (3), and Balls (4) in Gies' laboratory to which reference has already been made (5). Although their analytical methods were scarcely adequate to determine the very small amounts of aluminum present, still they constitute the most serious attempt of the time to answer the questions raised. In general it would appear, however, that their analytical data are quite too high, probably by reason of the inadequacy of the methods employed. Steel (2) carried out eleven experiments in which from 50 to 200 mg. of aluminum were administered to dogs either in the form of potassium aluminum sulfate or in baking powder biscuits. After 3 hours, blood was withdrawn and analyzed by the method of the Association of Official Agricultural Chemists, slightly modified (6) and found to contain 0.1 to 0.8 mg. of Al per 100 gm. of blood.[1] From this he concluded that "aluminum in comparatively large amounts promptly passed into the blood." A little later Kahn (3) conducted experiments on three dogs to which from 15 to 25 mg. of Al were administered daily in baking powder biscuits, employing the same analytical methods as Steel. He found as high as 11 mg. of Al per 100 gm. in bile and pancreas in individual instances with an average of 0.7 mg. of Al per 100 gm. of blood and 1.9 mg. per 100 gm. of liver. He concluded that aluminum passes in considerable

[1] When the data in the literature have been given in terms other than aluminum they have been recalculated.

amounts into the blood, accumulates to some extent in various parts of the body, and is excreted partly in both the bile and the urine. About 6 years later Balls (4) carried out analyses on the tissue of normal dogs and on dogs receiving aluminum, two animals being given aluminum in the form of the phosphate and two in baking powder biscuits. The Schmidt and Hoagland method (7) for aluminum was used. In the control group six samples of blood and a number of different tissues were analyzed, but with one exception, a sample of blood giving 0.05 mg. of Al per 100 gm. of blood, all of the results were regarded as being beyond the accuracy of the method. The blood of the aluminum-fed animals averaged about 0.1 mg. of Al per 100 gm., but wide variations were encountered in the aluminum content of the other tissues, the bile for example varying from 0.5 to 6.6 mg. of Al per 100 gm. Balls concluded: "Aluminum is absorbed by dogs from food containing aluminum phosphate, and from bread baked with alum baking powder. Much of this aluminum is speedily eliminated, some is retained, replacing part of the iron occurring normally in the tissues." In 1924 Killian (8) carried out tissue analyses with the Schmidt-Hoagland method on a dog which had received 190 mg. of aluminum daily for 7 weeks in the form of baking powder biscuits. The following results calculated in mg. of Al per 100 gm. of tissue were obtained: serum, 0.13; clot, 0.05; gallbladder and bile, 0.89; kidneys, 0.23; bones, 0.03. Before the aluminum diet was instituted the serum showed 0.04 mg. and the clot 0.06 mg. Although Killian did not regard the results dependable, owing to the minute amounts of aluminum found, his results show fair agreement with the figures reported in the present paper. Employing a modified Atack alizarin method Gray (9) obtained the following results on two normal dogs in individual or combined analyses calculated as mg. of Al per 100 gm. of tissue: blood 0.12, 0.14; kidney 0.60, 0.54; liver 0.50, 0.70; gallbladder 0.41; spleen 0.22; testicle 0.10; pancreas 0.10; bone marrow 0.72, figures which we believe too high in several instances.

EXPERIMENTAL.

The analytical method described in an earlier paper of this series (5) was applied to the tissues of normal dogs and dogs receiving aluminum. As a rule, when 40 gm. of tissue were available this amount was used for digestion, and color developments made on the equivalent of 12 gm. of tissue unless the aluminum content was known to be small, when larger amounts were employed. Most of the analyses on the normal dogs were made in triplicate, aluminum being added to one or two samples in each determination as a check on its recovery.

Aluminum Content of Tissues of Normal Dog.—With one exception the eight control dogs were obtained from the Department of Pharmacology after use in various experiments, in none of which

inorganic salts were employed. The animals had been fed on scraps obtained from the hospital diet kitchen.

A summary of these results is presented in Table I. It will be noted that the highest content of aluminum was found in the liver, followed by the heart, kidney, and spleen. In two other normal dogs (Nos. 9 and 10) 6.1 gm. and 5.3 gm. of bile were analyzed and gave 0.33 and 0.38 mg. of Al per 100 gm. respectively.

Aluminum Content of Tissues of Dog on Diet Containing Added Aluminum.—The animals used in this series of experiments were all mature dogs, procured from various sources. They were of no particular breed and their previous history is unknown. All of

TABLE I.

Aluminum Content of Tissue of Normal Dog.

Dog No.	Liver.	Heart.	Kidney.	Spleen.
	Mg. Al per 100 gm. tissue.			
1	0.16	0.10	0.06	0.07
2	0.18	0.12	0.07	0.08
3	0.10	0.10	0.11	0.07
4	0.31	0.17	0.14	0.03
5	0.10	0.11	0.06	
6	0.12	0.10	0.07	0.04
7	0.08	0.02	0.03	0.05
8	0.17	0.17	0.12	0.14
Average....	0.15	0.11	0.08	0.07

Aluminum figures of 0.04 mg. and below are regarded as too small to be reliable.

the animals, with the exception of Dog 11 which was accidentally killed by a puncture of the coronary artery while blood was being taken, were kept on the diet for approximately 90 days. The diet consisted of a cooked mixture of hashed beef (with some bone), corn-meal, and oatmeal to which a little salt was added. In addition to this stock diet Dogs 11 to 16 first received a sufficient number of biscuits raised with baking powder containing aluminum to be equivalent to 230 mg. of aluminum daily. Dogs 17 and 18 received 7 gm. of aluminum phosphate in their food daily, the aluminum phosphate being thoroughly incorporated in the stock diet, the amount of which was always less than the amount the

dogs consumed at one feeding. After the food containing the aluminum phosphate was eaten additional stock diet was given to satisfy each animal. All animals were fed once daily at 11 a.m. The animals ate well, were always healthy, and appeared to thrive on the diet.

At the completion of the diet period, the animals were killed by bleeding from the femoral artery while under ether anesthesia.

TABLE II.

Aluminum Content of Tissues of Dogs Receiving Aluminum.

Dog No. Weight, *kg*	11 8.5	12 14	13 14.3	14 15	15 11	16 17.6	17 16.2	18 15	Average.
Tissue or fluid.	Mg. Al per 100 gm. tissue or fluid.								
Blood			0.04	0.17	0.04	0.04	0.04	0.04	*
Bile		0.39	0.28	0.16	0.46	0.33	0.50	0.33	0.35
Liver	0.35	0.33	0.19	0.20	0.12	0.33	0.25	0.42	0.27
Intestine	0.43				0.27	0.18	0.32	0.35	0.31
Stomach	0.25								
Kidney	0.13	0.03	0.12	0.10	0.04	0.09	0.04	0.04	0.07
Urine			0.01	0.01		0.01		0.02	*
Heart		0.07	0.10	0.14	0.25	0.15	0.12	0.08	0.13
Muscle		0.21	0.10	0.13	0.10	0.12	0.05	0.17	0.12
Brain		0.18	0.04	0.04	0.05	0.09	0.12	0.16	0.10
Pancreas		0.12	0.13	0.10	0.12	0.11	0.08	0.04	0.10
Spleen	0.15		0.04	0.04	0.04	0.06	0.04	0.17	0.08
Lungs				0.08	0.12	0.17	0.04	0.08	0.10
Bone			0.04	0.04	0.04	0.04	0.04	0.04	*
Testes	0.14								

Dogs 11 to 16 received 0.23 gm. of aluminum daily for 90 days (excepting Dog 11 which was accidentally killed earlier) in biscuits raised with a baking powder containing aluminum, while Dogs 17 and 18 had 1.55 gm. of aluminum in the form of $AlPO_4$ daily incorporated in their food.

* Figures of 0.04 mg. and below are regarded as too small to be reliable.

No food was given the animals for 26 to 32 hours preceding the bleeding. The tissues were handled very carefully to prevent any contamination. The urine analyzed was obtained by catheter. The averages of duplicate analyses are summarized in Table II. Although the intake of aluminum in these animals was comparatively high, 17 mg. of Al per kilo for Dogs 11 to 16 and 105 mg. per kilo for Dogs 17 and 18, still there is relatively

little difference in the aluminum content of the tissues from that found in the control group, except in the case of the liver, where the average is 0.27 mg. instead of 0.15 mg. of Al per 100 gm. The figures for the bile, however, are the same for both the control dogs, Nos. 9 and 10, and Dogs 12 to 18 on the aluminum diet, namely 0.35 mg. per 100 gm. It is of some interest that the heart and muscle appear to contain slightly more aluminum than the brain, pancreas, or lungs, while the kidney contains less, and the figures obtained for the urine are too small to attach any significance to them.

Absorption and Excretion of Aluminum.—Several experiments have been carried out dealing with the absorption and excretion of aluminum. The absorption of aluminum salts was studied in one experiment from intestinal loops, two experiments were conducted on the time of elimination of injected aluminum, while two experiments were made to ascertain the excretion of injected aluminum by way of the bile and the urine.

For the intestinal loop experiments a dog weighing 10 kilos (Dog 19) was employed. A section of the intestine about 18 inches long was irrigated with warm physiological saline until the return was perfectly clear, then divided into two loops with the aid of broad tape ligatures so as to interfere as little as possible with the mesenteric circulation. Cannulæ were inserted through small incisions, tied in place, and 25 cc. of a warm solution of potassium aluminum sulfate in physiological saline injected. Unfortunately a part of the solution in the second loop was lost by a peristaltic contraction before it could be closed. The loops were replaced in the abdomen and the wound closed. After 3 hours the loops were removed. The aluminum had apparently precipitated on the mucosa in curdy lumps. The first loop was thoroughly cleaned and the aluminum content determined by the Schmidt-Hoagland method (7). The loop was found to contain 130.5 mg. of aluminum in comparison with 130.2 and 129.9 mg. determined in 25 cc. of the solution with the same method, thus indicating a complete lack of absorption.

The aluminum content of the tissues of two dogs was studied after the intravenous and intraperitoneal injection of potassium aluminum sulfate. The data are presented in Table III. It will be observed that a marked retention of aluminum existed after 8

days and that considerable aluminum was present in Dog 21 after the lapse of 34 days. The aluminum appears to have been well distributed, although the largest amounts were found in the abdominal muscle and skin. In harmony with the findings reported in the preceding paper (1) the figures for the aluminum content of the liver are very high. However, the aluminum

TABLE III.

Analysis of Tissue after Injection of Aluminum.

Tissue or fluid.	Dog 20.			Dog 21.		
	Amount digested.	Aliquot analyzed.	Al found.	Amount digested.	Aliquot analyzed.	Al found.
	gm.	*gm.*	*mg. per 100 gm.*	*gm.*	*gm.*	*mg. per 100 gm.*
Blood	54.0	5.4	0.18	40	12.0	0.06
Bile	12.5	12.5	0.32	9	9.0	0.44
Liver	40.0	1.6	2.25	40	3.2	0.87
Intestine	40.0	1.6	1.87			
Kidney	40.0	1.6	1.56	40	6.0	0.33
Heart	40.0	1.6	2.00	40	6.0	0.60
Abdominal muscle	40.0	0.8	4.38	20	2.0	1.15
" skin	31.0	0.6	5.64	20	2.0	0.90

Dog 20, a female weighing 8.6 kilos, was given intravenously 25 cc. of warm (37°) sterile physiological saline containing 5 mg. of Al in the form of potassium aluminum sulfate on Nov. 15 and 16, 1927, and intraperitoneally on each of the 12 following days, a total of 70 mg. of Al. Beyond the slight irritation from repeated punctures no ill effects were observed. The animal was killed by bleeding 8 days after the last injection.

Dog 21, a female weighing 10.6 kilos, was given 70 mg. of Al, the first 5 mg. injection being made intravenously on Nov. 16, 1927, while the remaining thirteen injections were given intraperitoneally on successive days. The animal showed no ill effects from the injections but 1 month later, possibly due to overheating and drafts in the animal house, developed a respiratory infection and died 3 days later, 34 days after the last injection.

content of the bile is practically normal, and thus furnishes no evidence of an increased excretion of aluminum in the bile. Unfortunately the specimens of urine were lost. In comparison with the observations on the diet animals it is significant that here appreciable amounts of aluminum were present in the blood of both dogs. The large amount of aluminum still present in the

body after periods of 8 and 34 days indicates a rather slow elimination of aluminum when once present in the body, and furnishes little support to the view that appreciable amounts of aluminum are absorbed from the alimentary tract.

In an attempt to demonstrate the excretion of injected aluminum in the bile two experiments have been performed. Two unfed female dogs, Dog 22 weighing 10.4 kilos, and Dog 23 weighing 9 kilos, were given 1.5 gm. of urethane per kilo of body weight. As soon as the urethane had taken effect, ether was given, a tracheal cannula inserted, and the gallbladder exposed by a median incision of the abdominal wall. The gallbladder was ligated and the common bile duct cannulated from the lumen of the intestine.

TABLE IV.

Influence of Intravenous Administration of Aluminum upon Aluminum Content of Bile and Urine.

Animal No.	Fluid.	Fluid obtained and digested.	Aliquot used.	Al found.
		gm.	*gm.*	*mg. per 100 gm.*
22	Gallbladder bile.	5.3	5.3	0.38
	Cannula "	7.5	2.25	0.44
	Urine.	15.0	4.5	0.67
23	Gallbladder bile.	Lost.		
	Cannula "	14.6	14.6	0.23
	Urine.	20.0	5.0	0.40

The cannula bile was collected over a period of 3½ hours in Dog 22 and 4½ hours in Dog 23.

30 cc. of 0.35 per cent hydrochloric acid were injected into the intestine posterior to the opening of the bile duct to stimulate the flow of bile, and the bile collected. The femoral vein was next exposed and 100 cc. of warm (37°) physiological saline containing 10.84 mg. of aluminum as aluminum chloride were injected. A catheter was also inserted into the urinary bladder and the urine collected.

At the close of the experiment the ligated gallbladder was removed and the contents analyzed as normal bile.

The data obtained are summarized in Table IV. It seems hardly proper to compare the cannula bile with the bladder bile since the latter is normally 6 to 10 times more concentrated.

However, in Dog 22 the cannula bile is found to be slightly richer in aluminum. The figures for the urine are so high that they would suggest the kidney as one path for the excretion of aluminum, at least when appreciable amounts are present in the blood.

DISCUSSION.

Traces of aluminum are present in the tissues of the normal dog. Of the tissues analyzed, heart, kidney, spleen, and liver, the latter contained the largest amount, averaging in eight animals 0.15 mg. of Al per 100 gm. The administration of relatively large amounts of aluminum has practically no influence upon the aluminum content of the heart, kidney, spleen, and bile, although in the case of the liver there appears to be a very slight but definite influence, the average figure for eight animals being 0.27 mg. of Al per 100 gm. The figures obtained for the aluminum content of the blood are so small as to be within the limits of experimental error of the method.

When a small amount of aluminum (70 mg.) was administered to dogs intravenously and intraperitoneally in 5 mg. daily portions, it apparently remained in the tissues for a long time after the last injection, since very high figures were found at the end of 8 days, and after 34 days the amount was still 3 or more times that found in the aluminum diet animals. In the one experiment in which the question of absorption of aluminum from an intestinal loop was studied complete recovery of aluminum was obtained.

The data presented in this paper, we believe, speak strongly against the absorption of more than traces of aluminum from the alimentary tract. If this view is incorrect, it is hard to comprehend why small amounts of injected aluminum should be retained in the tissues for a long time, when very large quantities given by mouth daily (45 to 300 times as much) have only a very slight influence. In other words, if aluminum is poorly eliminated when parenterally introduced, it is illogical to believe that when orally introduced it should be absorbed and rapidly eliminated. To be sure we have observed an appreciable excretion in the urine when aluminum chloride was introduced intravenously (see Table IV), but if a significant amount of ingested aluminum were normally eliminated in this way, this fact would not have been overlooked by the Referee Board (10) and by Schmidt and

Hoagland (11), nor would they have obtained complete recovery in the feces of the ingested aluminum. We do not feel, therefore, that our observations lend any support to the contention of Steel (2), Kahn (3), and Balls (4), that considerable quantities of aluminum are absorbed from the digestive tract of the dog when aluminum is ingested in the food.

There are three possible paths for the elimination of aluminum when it is present in the body tissues and fluids: the bile, the urine, and the skin. Kahn (3) concluded that aluminum was excreted to some extent in both the bile and the urine, which is probably correct. Our data do not appear to give a definite answer as to which is the chief path of excretion, and more work is needed on this point. That some aluminum may be excreted in the urine after the injection of aluminum would seem evident from the data presented in this and the preceding paper. If the kidney is the most important path for the excretion of aluminum it does not retain the aluminum long as the aluminum content of this tissue is relatively low. We have been inclined to regard the excretion through the bile as the most important owing to the higher concentration of aluminum in the liver and the bile than in the other tissues and fluids, but the fact that the dogs receiving aluminum in their food or by injection showed essentially the same concentration of aluminum in the bile as the normal dogs does not strengthen this view.

The dogs receiving aluminum by intraperitoneal injection showed the highest content of aluminum in the abdominal muscle and skin, but this may well have been influenced by the site of administration.

SUMMARY.

Aluminum is normally present in such tissues of the dog as the spleen, kidney, heart, and liver in amounts varying from 0.07 to 0.15 mg. per 100 gm., the largest amounts being present in the liver.

After the daily ingestion of 0.23 and 1.55 gm. of aluminum (six and two animals respectively) for 3 months, there was practically no change in the aluminum content of these same tissues, with the exception of the liver, where the amount averaged 0.27 mg. per 100 gm. The aluminum content of the bile of these

animals and two control animals averaged 0.35 mg. in both groups. The figures obtained for the aluminum content of the blood of the aluminum diet animals were so small as to be within the limits of experimental error of the method.

When 5 mg. of aluminum were administered parenterally to two dogs daily for 2 weeks, a marked increase in the aluminum content of the tissues was found 8 and 34 days after the administration of aluminum had been discontinued, indicating that when aluminum is present in the tissues it is slowly excreted.

These data lead to the conclusion that when aluminum compounds are administered orally to dogs the absorption of the aluminum is very slight.

The excretion of aluminum when present in the body tissues and fluids is discussed.

BIBLIOGRAPHY.

1. Myers, V. C., and Mull, J. W., *J. Biol. Chem.*, 1928, lxxviii, 605.
2. Steel, M., *Am. J. Physiol.*, 1911, xxviii, 94.
3. Kahn, M., *Biochem. Bull.*, 1911–12, i, 235.
4. Balls, A. K., *Biochem. Bull.*, 1916, v, 195, and unpublished dissertation, Columbia University, New York, 1917.
5. Myers, V. C., Mull, J. W., Morrison, D. B., *J. Biol. Chem.*, 1928, lxxviii, 595.
6. Steel, M., *Biochem. Bull.*, 1916, v, 173.
7. Schmidt, C. L. A., and Hoagland, D. R., *J. Biol. Chem.*, 1912, xi, 387.
8. Killian, J. A., unpublished analyses cited by Smith, E. E., Aluminum compounds in food, New York, 1928, 228.
9. Gray, P. B., cited by Smith, p. 229.
10. Referee Board of Consulting Scientific Experts, *U. S. Dept. Agric., Bull. 103*, 1914.
11. Schmidt, C. L. A., and Hoagland, D. R., *Univ. Calif. Pub. Path.*, 1919, ii, 215.

THE ALUMINUM CONTENT OF HUMAN AUTOPSY TISSUE.

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Comparatively little attention appears to have been given to the aluminum content of human tissue, although a few analyses have been reported by Gonnermann (1, 2) and Keilholtz (3), the former obtaining relatively high values and the latter negative results. We have thought it desirable to present the few observations which we have made on human tissue at this time. On several occasions attempts have been made to estimate the aluminum content of human blood (4), on samples of from 5 to 30 cc., but the amounts of aluminum present appear to be too small to estimate satisfactorily.

The method previously described (5) has been applied to human autopsy material, kindly furnished us at the Pathological Laboratory of the City Hospital. Our analytical data are presented in Table I. As will be noted, the magnitude of the figures is quite comparable with those found in the rat (6) and dog (7). In contrast with the control analyses on the rat and dog, however, the figures for the liver appear to be much lower, whereas the aluminum content of the brain and heart is considerably higher. We do not wish to draw any definite conclusions from the limited number of observations recorded on human tissue, but we have thought it desirable to present the data available in conjunction with our findings on the rat and dog.

BIBLIOGRAPHY.

1. Gonnermann, M., *Biochem. Z.*, 1918, lxxxviii, 401.
2. Gonnermann, M., *Z. physiol. Chem.*, 1920, cxi, 32.
3. Keilholtz, A., *Pharm. Weekbl.*, 1921, lviii, 1482.
4. Mull, J. W., Morrison, D. B., and Myers, V. C., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 476.

5. Myers, V. C., Mull, J. W., and Morrison, D. B., *J. Biol. Chem.*, 1928, lxxviii, 595.
6. Myers, V. C., and Mull, J. W., *J. Biol. Chem.*, 1928, lxxviii, 605.
7. Myers, V. C., and Morrison, D. B., *J. Biol. Chem.*, 1928, lxxviii, 615.

TABLE I.

Aluminum Content of Human Autopsy Tissue.

Case No.	Diagnosis.	Brain.	Heart.	Liver.	Gall bladder and bile.	Kidney.	Spleen.
		Mg. Al per 100 gm. tissue.					
1	Peritonitis		0.27	0.05	0.05	0.02	
2	Acute alcoholism	0.14					
3	Bronchopneumonia	0.45	0.13	0.10		0.06	0.05
4	Diphtheria			0.05	0.03		
5	Advanced pulmonary tuberculosis	0.36			0.10	0.10	
6	Carcinoma of esophagus		0.14	0.09	0.17	0.06	0.13
7	Pulmonary tuberculosis		0.25		0.01		
8	Tubercular bronchopneumonia		0.23	0.12		0.20	0.12
9	Typhoid ulceration	0.07					0.03
10	Cardiorenal disease	0.20					
11	Acute splenic hyperplasia, *etc.*	0.22		0.07			
12	Heart failure	0.12	0.22	0.14		0.05	
13	" " pulmonary tuberculosis	0.35	0.29			0.34	0.07
14	Bronchopneumonia		0.11				0.05
No. of specimens		8	8	7	5	7	6
Average		0.25	0.21	0.08	0.07	0.10	0.07

ON THE SEPARATION OF HISTIDINE AND ARGININE.

IV. THE PREPARATION OF HISTIDINE.*

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Histidine was discovered simultaneously by Kossel (1) and by Hedin (2). Kossel first obtained it by precipitation with mercuric chloride from the alkaline solution containing the products of hydrolysis of the protamine, sturine. Hedin isolated it from the precipitate formed when the base fraction from hydrolyzed protein was treated with silver nitrate and then with ammonia until a maximum precipitation occurred. Both methods were subsequently further developed by Kossel and his associates, but for purposes of large scale preparation the mercuric chloride method has been almost universally employed. Fränkel (3) in 1903 suggested that hemoglobin was the most suitable source of histidine and described its preparation by precipitation with mercuric chloride from a solution maintained alkaline with sodium carbonate. Modifications of this procedure have been repeatedly described (4–8), but it is clear, from the pains that have been taken by different workers to define the exact conditions for success, that much difficulty has been encountered in obtaining good yields of a pure product.

While we have, as yet, had no occasion to study in detail the precipitation of amino acids by mercuric chloride in alkaline solution, the fact that lysine may be precipitated in this way (9, 10) indicates that the procedure is not necessarily as selective for histidine as has been supposed. We have therefore felt that

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

some of the other precipitants for histidine should be investigated with the object of developing a method for the preparation of this base which might be less subject to unaccountable variations in the quantity and quality of the product ultimately obtained.

Hedin's method for precipitating histidine by the use of silver was developed by Kossel and Kutscher (11) into a quantitative method of analysis and the further step of precipitating the histidine by Hopkins' mercuric sulfate reagent, subsequently advocated by Kossel and Patten (12) and by Osborne, Leavenworth, and Brautlecht (13), added greatly to the accuracy of this method.

As we have pointed out in a previous paper (14) histidine is completely precipitated when an excess of silver ion is added to a protein hydrolysate and the reaction is brought to pH 7.0 by the addition of barium hydroxide. The convenience of a direct separation of histidine in this way from most of the other products of hydrolysis of the protein is apparent and, while silver precipitation at pH 7.0 is by no means highly selective, it appears that very little in addition to histidine that is so precipitated is subsequently thrown down by mercuric sulfate. The removal of the small proportion of impurity is easily effected by suitable treatment of the final product. This method is, in principle, similar to Kossel and Patten's (12) method for the determination of histidine, and the more nearly quantitative nature of the precipitations render it, in our estimation, more suitable for the preparation of this base than the customarily employed mercuric chloride-sodium carbonate method. By proper attention to details of technique the labor involved in the operations can be reduced to a minimum.

The general procedure is as follows: (1) The protein (hemoglobin or washed and coagulated blood cells) is hydrolyzed and the greater part of the acid removed. (2) An excess of silver oxide suspended in water is added, the acidity of the solution being maintained by the addition of sulfuric acid as required but any material excess should be avoided. (3) The solution is brought to pH 7.4 by the addition of barium hydroxide solution and the precipitate, which contains the histidine together with other amino acids, is filtered off and washed. (4) The precipitate is suspended in water, sulfuric acid sufficient to give a faint reaction to Congo

red is added, and hydrogen sulfide is then passed in until the precipitate is completely decomposed. The silver sulfide is centrifuged off and the solution is concentrated to remove hydrogen sulfide. (5) The solution is made to contain 5 per cent of sulfuric acid, treated with Hopkins' mercuric sulfate reagent in excess, and allowed to stand at least 24 hours. The precipitate is filtered off, washed with diluted reagent, and decomposed with hydrogen sulfide. The mercuric sulfide is removed, the solution is concentrated slightly to expel hydrogen sulfide and is then treated with sufficient of a solution of recrystallized barium hydroxide to bring the reaction to pH 7.2 and filtered. (6) The perfectly clear filtrate is, if necessary, boiled with a little norit and concentrated *in vacuo* until a part of the histidine has separated in crystalline form. The flask is removed from the still and the contents heated until all is again in solution, and approximately an equal volume of warm absolute alcohol is added. After the mixture has stood in ice overnight, crude histidine separates very completely and is filtered off and washed with 80 per cent and then with absolute alcohol. A second small crop may be obtained by concentration of the mother liquors and similar treatment.

If further purification is desired this material may be converted to the dichloride and crystallized and free histidine recovered as described below.

Discussion of the Procedure.

The best material for the preparation of histidine is hemoglobin which can be very easily prepared in crystalline form by the method of Ferry and Green (15). Failing this, crude blood cells are probably the most readily available and cheapest source. We have found it useful to prepare a stock of dry material. Fresh cattle blood (22 liters) was diluted with an equal volume of 1 per cent sodium fluoride solution and centrifuged. The serum was drawn off as closely as possible and the pulp of cells diluted with an equal volume of 0.9 per cent sodium chloride solution and again centrifuged. The pulp was then slowly poured into a large volume of boiling distilled water, the coagulum strained off, pressed, ground, and washed with liberal amounts of hot water, pressed, ground, and extracted with 80 per cent alcohol, and finally dried and ground to a fine powder. Yield 2800 gm., nitrogen 16.7 per

cent ash- and moisture-free. This material, while it contains appreciable amounts of serum proteins is largely hemoglobin and is an excellent source of histidine.

Hydrolysis of the protein may be effected either with 6 N hydrochloric acid or with 8 N sulfuric acid. For most of this work we have used hydrochloric acid, but serious losses of histidine may occur during the removal of silver chloride unless this is extracted with hot dilute hydrochloric acid and the extract subsequently worked over. We therefore recommend the use of sulfuric acid. If the barium sulfate is washed by decantation as described below, very little labor is required.

The single experiment in which hydrolysis was effected in an autoclave as described in a previous paper (14) was not satisfactory as it was found very difficult to secure a pure product. This method of hydrolysis is therefore not to be recommended for the preparation of histidine although further investigation may show how it may be improved.

The addition of an excess of silver oxide to the solution maintained slightly acid to Congo red or brom-phenol blue usually presents no difficulty unless the acidity is allowed to become too great. We have occasionally worked with solutions which failed to give the brown spot test with barium hydroxide although silver sulfate was separating in crystalline form on the walls of the vessel. If such a solution is carefully treated with barium hydroxide solution until the reaction is only faintly acid to brom-phenol blue paper, it will usually show the presence of excess silver after the addition of a little further silver oxide.

We have not carried through this preparation using silver nitrate instead of the oxide. Previous experience with the nitrate has shown, however, that no difficulties are to be expected. The silver precipitate obtained under these conditions should be rather more thoroughly washed than is necessary when the oxide is used.

The precipitation at pH 7.4 instead of at pH 7.0, which we have previously recommended, is necessary when one is dealing with a protein rich in histidine since, otherwise, a little histidine escapes precipitation.

The precipitation with Hopkins' reagent calls for little comment. The solution should have a volume of about 1 liter for

each 10 gm. of histidine expected and such a solution usually requires about 1½ volumes of the reagent for complete precipitation. Mercuric sulfate solution should be added until a clear sample of the fluid gives no more precipitate when a little reagent is added and the sample is allowed to stand for some time. The amount of histidine left in the filtrate from such a precipitation is insignificant.

Although in his first paper on the subject Kossel showed that free histidine could be readily obtained in crystalline form, most workers have made use of the salts, particularly the mono- and dihydrochlorides. The ease with which the free base may be brought to crystallization at its isoelectric point (16) suggested that it might advantageously serve for isolation. Unfortunately the crude histidine so obtained is contaminated with monoamino acids among which tyrosine has been positively identified. The material usually contains approximately 25 per cent of nitrogen (theory 27.1 per cent) but for some purposes such a preparation, which is free from other bases, is sufficiently pure. In order to secure analytically pure histidine it is necessary to carry out the crystallization of a salt since, owing to the nature of the impurities, the recrystallization of the crude material is ineffective.

EXPERIMENTAL.

Hydrolysis.—500 gm. of dry coagulated blood cells were treated with 2.5 liters of 8 N sulfuric acid (567 cc. of concentrated sulfuric acid + 2100 cc. of water) and heated in a boiling water bath with frequent shaking for several hours. The material was then boiled for 30 hours and poured into about 15 liters of distilled water. Approximately 95 per cent of the calculated amount of crystallized barium hydroxide was weighed out (1 cc. of concentrated sulfuric acid requires 5.56 gm.), dissolved in boiling water, and added in a thin stream with constant stirring. The barium sulfate was allowed to settle and the clear fluid drawn off. The barium sulfate was washed twice by decantation with a liberal amount of water and finally centrifuged. The clear fluids were concentrated either in a large vacuum still, or by boiling in open dishes, to 6 liters. A small amount of silver oxide suspension was added and the trace of silver chloride (derived from the chloride contamination of the material employed) and any remaining barium sul-

fate removed by filtration through paper pulp. An excess of silver oxide was then added as shown by the formation of a buff-colored precipitate when a drop was treated with a drop of cold barium hydroxide solution, and the solution was brought to pH 7.4 by the addition of warm saturated barium hydroxide solution.[1] The precipitate was filtered off and thoroughly washed with water on the funnels. It was then suspended in water, disintegrated by brushing through silk bolting cloth, acidified with sulfuric acid, and treated with hydrogen sulfide on a shaking machine. The precipitate was removed and washed and the fluid concentrated to approximately 2.5 liters. Concentrated sulfuric acid to make 5 per cent was added and the histidine precipitated by the addition of 4.5 liters of Hopkins' reagent. After standing 2 days the precipitate was removed and washed, suspended in water, acidified with sulfuric acid, and decomposed with hydrogen sulfide. The mercuric sulfide was removed and washed and the solution concentrated. A solution of recrystallized barium hydroxide was added until the reaction was at pH 7.2. As it is inadvisable to return the test samples containing indicator to the main solution, the most economical method of adjusting the reaction is by comparison on a porcelain plate of the color of single drops to which a dilute solution of indicator has been added with drops of appropriate buffer solutions. A final test may be made on a small sample which has been centrifuged clear. The isoelectric point of histidine is not known with sufficient accuracy to necessitate closer control than can readily be obtained in this way.

The solution was filtered, concentrated *in vacuo* to approximately 500 cc., boiled with norit, and further concentrated until a copious separation of crystalline histidine occurred. The flask was removed from the still, heated until the histidine had redissolved, and the contents (180 cc.) were washed with a minimal amount of hot water into a beaker. An equal volume of hot absolute alcohol was added. Under these conditions histidine separates promptly and very completely when allowed to stand in ice overnight. A small second crop can be secured from the mother liquor.

Crude histidine may be prepared in this way in a yield of be-between 4 and 5 per cent of the blood cells hydrolyzed. It

[1] For additional details see Vickery and Leavenworth (14, 17).

contains a little tyrosine and probably other amino acids. A typical first crop weighing 21 gm. decomposed at 268–270° and was ash-free. This indicated a high degree of purity but its nitrogen content was 25.24 per cent (theory 27.10 per cent) and Millon's reaction was faintly positive. Recrystallization from 300 cc. of 50 per cent alcohol gave 16 gm. with a nitrogen content of 25.56 per cent, indicating very little purification although the material was uniform and well crystallized and decomposed as before at 268°. Evidently the decomposition point of histidine cannot be used as an index of purity.

Purification of Crude Histidine.—A sample of crude histidine weighing 26.7 gm. (N 24.4 per cent) was dissolved in 150 cc. of water with the aid of a little hydrochloric acid and boiled with norit. The solution was then concentrated *in vacuo* to 40 cc. and transferred, by the use of 40 cc. of concentrated hydrochloric acid, to a beaker. After it was chilled, 80 cc. of alcohol saturated with hydrogen chloride were added. Crystallization occurred at once and, after it had stood in ice 3 hours, 34 gm. of dihydrochloride were obtained by filtration on a sintered glass funnel and drying over lime *in vacuo*. This crop contained 17.69 per cent of nitrogen (theory 18.43 per cent) and was therefore still slightly impure. It was recrystallized from a mixture of 20 cc. of water, 20 cc. of concentrated hydrochloric acid, and 40 cc. of alcoholic hydrogen chloride, when 31 gm. with a nitrogen content of 18.35 per cent were secured. This is equivalent to 21 gm. of free histidine or 78 per cent of the crude histidine taken. When histidine dihydrochloride is crystallized under these conditions it is not worth while to attempt to secure second crops.

A small quantity of tyrosine was isolated from the mother liquor of this second crystallization of the histidine dichloride. The reagents were removed and a small amount of typical needles giving Millon's reaction and containing 8.0 per cent of nitrogen (theory 7.74 per cent), was secured by direct crystallization. The mother liquor of the tyrosine apparently contained a mixture of amino acids from which nothing definite could be separated.

The recovery of free histidine from the dihydrochloride may be conveniently effected as follows, although the yield leaves something to be desired. 20.3 gm. of dihydrochloride were dissolved in 200 cc. of water and heavy magnesium oxide was added to the

boiling solution until it was neutral to litmus. The solution was then promptly filtered. Further small amounts of magnesia were then added until the solution was slightly more alkaline than pH 7.2. The solution was boiled with norit, filtered, the reaction adjusted to pH 7.2 with dilute hydrochloric acid, and concentrated to 90 cc. An equal volume of absolute alcohol was added and, when the solution was chilled for 24 hours, 10.4 gm. of free histidine separated. The mother liquor on evaporation to 20 cc. and treatment as before with alcohol yielded 0.9 gm. more. Together these crops represent 81 per cent of the histidine in the dihydrochloride used. Once recrystallized the product was analytically pure.

Recovery of Silver.—Inasmuch as the use of ammonia for the recovery of silver residues is objectionable for a number of reasons, among them the possibility of the formation of explosive compounds, we have discarded the method described in a former paper (14) and are now making use of the method described by Robertson (18). We have found this perfectly satisfactory even when the silver chloride contains considerable barium sulfate. The mixture of metallic silver and barium sulfate, obtained by reduction with glucose, is heated in a large silica dish to drive off organic impurities and then warmed with dilute nitric acid until all the silver is dissolved. The barium sulfate may then be removed by filtration. We have also found it possible, although not particularly advantageous, to recover silver sulfide directly by this method without previously oxidizing it to sulfate with nitric acid. It is necessary to adhere to the concentration of sodium hydroxide solution advocated by Robertson and to use a grade of sodium hydroxide free from iron as otherwise the silver oxide ultimately prepared is apt to be contaminated with this metal.

SUMMARY.

A method for the preparation of histidine from hemoglobin, or from coagulated blood cells, is described, consisting of four steps: (1) hydrolysis of the protein, (2) precipitation of histidine as its silver compound at pH 7.4, (3) precipitation of histidine with Hopkins' mercuric sulfate reagent, (4) direct crystallization of crude histidine as free base at its isoelectric point. The product

may be finally purified, with little loss, by crystallization of the dihydrochloride. A yield of from 4 to 5 per cent of the dry blood cells is readily secured.

BIBLIOGRAPHY.

1. Kossel, A., *Z. physiol. Chem.*, 1896, xxii, 176.
2. Hedin, S. G., *Z. physiol. Chem.*, 1896, xxii, 191.
3. Fränkel, S., *Monatsh. Chem.*, 1903, xxiv, 230.
4. Knoop, F., *Beitr. chem. Physiol. u. Path.*, 1907, x, 111.
5. Brigl, P., *Z. physiol. Chem.*, 1910, lxiv, 337.
6. Jones, H. M., *J. Biol. Chem.*, 1918, xxxiii, 429.
7. Demjanowski, S., *Z. physiol. Chem.*, 1922, cxxii, 93.
8. Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 521.
9. Vickery, H. B., *J. Biol. Chem.*, 1924, lxi, 117.
10. Winterstein, E., *Z. physiol. Chem.*, 1905, xlv, 77.
11. Kossel, A., and Kutscher, F., *Z. physiol. Chem.*, 1900, xxxi, 165.
12. Kossel, A., and Patten, A. J., *Z. physiol. Chem.*, 1903, xxxviii, 39.
13. Osborne, T. B., Leavenworth, C. S., and Brautlecht, C. A., *Am. J. Physiol.*, 1908, xxiii, 180.
14. Vickery, H. B., and Leavenworth, C. S., *J. Biol. Chem.*, 1927, lxxv, 115.
15. Ferry, R. M., and Green, A. A., in preparation.
16. Kanitz, A., *Z. physiol. Chem.*, 1906, xlvii, 476.
17. Vickery, H. B., and Leavenworth, C. S., *J. Biol. Chem.*, 1927, lxxii, 403.
18. Robertson, G. R., *J. Am. Chem. Soc.*, 1927, xlix, 2884.

THE RELATION OF COPPER TO THE HEMOGLOBIN CONTENT OF RAT BLOOD.*

PRELIMINARY REPORT.

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(Received for publication, May 19, 1928.)

In previous publications by the senior author (1–9) it has been shown that copper, manganese, and zinc are normal constituents of plants and animals, and that these elements occur in greater concentration in the livers of domestic animals than in any other organ. It has also been found that manganese, copper, and zinc occur in greatest concentrations in those organs of animals that have the greatest vitamin potency, and furthermore it has been shown that the livers of calves at the time of birth contain more copper than do the livers of more mature animals of the same species.

Porter (10) in 1875 published a text-book on chemistry in which he states that copper is a normal constituent of human blood. However, it has not been previously demonstrated that copper has an important function in red blooded animals, although numerous observations have been published from time to time which indicate that copper has a respiratory function in those animals that have colorless and blue blood.

The following experiment was performed to ascertain the copper content of the serum and cells of fresh blood of a mature domestic animal. 5 quarts of fresh blood were obtained from a normal 2-year-old heifer which was slaughtered for beef. The fresh, warm blood was shaken with glass beads to separate the fibrin and cells from the serum. The mixed mass was centrifuged

* Read before the Division of Biological Chemistry at the Seventy-Fifth meeting of the American Chemical Society, St. Louis, April 16–20, 1928.

to free further the serum from the cells and fibrin. The clear serum was decanted from the mass of blood cells, evaporated, and dried at about 100°. The mass of cells and fibrin still containing

TABLE I.

Analysis of Cow Blood.

	Dry serum.	Dry cells and fibrin.
	per cent	*per cent*
Copper	0.0044	0.0007
Iron	0.0140	0.1580
Manganese	Trace.	Trace.
Zinc	0.0096	0.010
Phosphorus	0.1420	0.0798
Calcium	0.1120	0.0256
Magnesium	0.145	0.309
Potassium	0.275	0.112
Sodium	3.53	1.151
Nitrogen	12.80	15.28
Total sulfur	1.544	0.579

TABLE II.

Analysis of Calf Liver.

	Moisture-free.
	per cent
Copper	0.0125
Iron	0.0358
Manganese	0.0003
Zinc	0.040
Phosphorus	1.1218
Calcium	0.028
Magnesium	0.161
Potassium	0.896
Sodium	0.772
Nitrogen	10.24
Protein	64.00
Total sulfur	0.584

some serum was dried in a similar manner. Each of the two portions was carefully ashed in silica dishes and analyzed with the results shown in Table I.

In order to demonstrate whether or not the copper contained in red blood has any useful function, as its presence suggests, livers of young calves were obtained, dried, ground, and analyzed. With other portions of the livers the following experiment was performed with white albino rats. The analysis of the dry calf livers is given in Table II.

TABLE III.

Hemoglobin Content of Blood of Rats without and with Copper in Their Diet.

Lot 1.	Solution of ash of calf liver, copper removed.	
	Tallqvist method.	Fleischl method.
Mar. 28, 1928.		
Rat 1	75	66
" 2	65	68
" 3	65	76
" 4	75	86
Average.......	70	74
Apr. 3, 1928.		
Rat 1	68	70
" 2	68	80
" 3	68	83
" 4	70	78
" 5	75	77
" 6	68	74
" 7	75	86
" 8	74	83
" 9	68	68
" 10	74	80
Average.......	70.8	77.9

Lot 2.	Solution of ash of calf liver.	
	Tallqvist method.	Fleischl method.
Mar. 28, 1928.		
Rat 1	95	90
" 2	90	110
" 3	90	85
Average.......	91.7	95
Apr. 3, 1928.		
Rat 1	95	87
" 2	100	104
" 3	90	85
" 4	90	90
" 5	78	87
Average........	90.6	90.6
Apr. 4, 1928.		
Rats + 2 gm. dry calf liver per day.		
Rat 6	90	87
" 7	85	89
" 8	80	70
Average........	85	82

Two equal portions of the moisture-free liver were ashed separately in quartz dishes, the ash treated with hydrochloric acid, evaporated to dryness, taken up with a few drops of hydrochloric acid and a little water, and diluted with water to such a volume that 1 cc. contained approximately 0.1 mg. of iron. The HCl solution of the other portion of liver was treated with H_2S to precipitate the copper it contained, and the precipitate filtered out

and washed. The filtrate from the copper precipitate was evaporated to dryness with a few drops of nitric acid to oxidize ferrous iron to the ferric condition. Hydrochloric acid was added to the residue and evaporated to a pasty consistence and this operation was repeated twice, after which water was added to make the volume the same as the solution which contained copper.

Two lots of white albino rats, each rat weighing about 50 gm., were put into wire cages fitted with screen floors, two rats in each cage. The rats were fed skim milk in the morning and whole milk in the afternoon. The milk was placed in small glass tumblers attached inside the cages. Distilled water was also available to the rats, *ad libitum*. After the rats had been on the milk diet for about 4 weeks, 10 cc. of the solution without copper were added to the portion of skim milk given to one lot of rats once each day, and a like amount of the solution containing copper was added to the skim milk given to the second lot of rats once each day.

At the end of the 6th week, hemoglobin determinations were made from the blood of the two lots of rats, with the results shown in Table III.

The hemoglobin readings were made independently by Healy and McHargue, the average reading being taken. The analyses of the blood serum and cells and the liver were made by Hill.

When the samples of blood for the hemoglobin determinations were taken, it was observed that the blood from the rats from which copper was withheld was noticeably darker in color than the blood from the rats that received copper, the latter's blood being a brighter red. The samples of blood were obtained by clipping off sections of the tails of the rats, discarding the first few drops, and collecting the blood by means of a Fleischl blood pipette. Drops of fresh blood were absorbed on pieces of standard filter paper and immediately matched with the standard color chart in the Tallqvist method.

The rats that received copper in their diet possessed to a noticeable degree a pink ruddiness where the skin is exposed, for example on the tail, feet, and nose, which was absent on those from which copper was withheld.

From the external appearance of the rats, the color of the fresh blood, together with the hemoglobin readings, the authors infer

that copper has an important function in the formation of hemoglobin and in the metabolism of animals having red blood.

Further experiments concerning the effect of copper, manganese, zinc, *etc.*, are under way.

Addendum.—Since this article was submitted for publication Hart, Steenbock, Waddell, and Elvehjem have published similar findings (11).

BIBLIOGRAPHY.

1. McHargue, J. S., The association of manganese with vitamins, *J. Agric. Research*, 1924, xxvii, 417.
2. McHargue, J. S., The occurrence of copper, manganese, zinc, nickel and cobalt in soils, plants and animals and their possible functions as vital factors, *J. Agric. Research*, 1925, xxx, 193.
3. McHargue, J. S., The association of copper with substances containing the fat-soluble A vitamin, *Am. J. Physiol.*, 1925, lxxii, 583.
4. McHargue, J. S., The significance of the occurrence of copper, manganese and zinc in forage crops and foods, *J. Am. Soc. Agron.*, 1925, xvii, 368.
5. McHargue, J. S., Manganese and plant growth, *Ind. and Eng. Chem.*, 1926, xviii, 172.
6. McHargue, J. S., Further evidence that small quantities of copper, manganese and zinc are factors in the metabolism of animals, *Am. J. Physiol.*, 1926, lxxvii, 245.
7. McHargue, J. S., Significance of the occurrence of manganese, copper, zinc, nickel and cobalt in Kentucky bluegrass, *Ind. and Eng. Chem.*, 1927, xix, 274.
8. McHargue, J. S., The proportion and significance of copper, iron, manganese and zinc in some mollusks and crustaceans, *Kentucky Acad. Sc.*, 1927, ii, 46.
9. McHargue, J. S., Metals in plants, their importance to humans, *Research Narratives*, 1927, vii.
10. Porter, J. A., Principles of chemistry, improved edition, New York and Chicago, 1875, 333.
11. Hart, E. B., Steenbock, H., Waddell, J., and Elvehjem, C. A., *J. Biol. Chem.*, 1928, lxxvii, 797.

A MICRO METHOD FOR THE DETERMINATION OF POTASSIUM AS IODOPLATINATE.*

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(Received for publication, June 6, 1928.)

The standard and most widely accepted method for the determination of potassium depends upon its precipitation as potassium chloroplatinate. The modification called the Lindo-Gladding procedure (1) is official with the Association of Official Agricultural Chemists. Methods based upon the precipitation of potassium as potassium of cobalti-nitrite (2), have been very useful in determination of potassium in the blood and serum when the technique described (3) is rigidly adhered to, but under slightly different conditions, or for material of varying salt composition, are questionable. This criticism applies also to modifications involving either the gasometric (4) or colorimetric (5) determination. The cobalti-nitrite precipitate is not of constant composition (6).

The method about to be presented depends upon the precipitation of the potassium chloroplatinate in the ash of physiological material and its subsequent determination by colorimetric or titrimetric procedure or by both.

The quantitative determination depends upon the conversion of potassium chloroplatinate to potassium iodoplatinate by the addition of potassium iodide. Because of the deep rich wine color of the iodoplatinate in solution, amounts as small as 0.1 mg. of potassium in this form can be estimated by colorimetric comparison with known amounts of potassium chloroplatinate also treated in this way. The principle was used in 1903 by Cameron and Failyer (7) for the determination of potassium in water. The presence of iodine in the compound permits the use of the

* Read before the American Society of Biological Chemists, 1927, at the Rochester Meeting (*J. Biol. Chem.*, 1927, lxxix, p. iv).

method of Peterson (8), in which potassium iodoplatinate is reduced in neutral solution to K_2PtI_4 with sodium thiosulfate.

The probable reactions taking place are expressed in the following equations.

$$K_2PtCl_6 + 6KI = K_2PtI_6 + 6KCl$$
$$K_2PtI_6 + 2Na_2S_2O_3 = K_2PtI_4 + 2NaI + Na_2S_4O_6$$
$$(Pt^{++++} + 2S^{++} = Pt^{++} + 2S^{+++})$$

The color varies in proportion to the amount of potassium chloroplatinate present when potassium iodide is added to solutions of this salt. This proportion is maintained from 0.2 mg. per cent up to 10.0 mg. per cent, which is the highest concentration tried. Dilution does not affect the proportion. The color given by 1.6 mg. per cent and more cannot be read accurately in the colorimeter without previous dilution.

The rate of color development is proportional to the amount of potassium iodide present. The presence of acid also hastens color development, but after a time excess acid causes the liberation of iodine. The presence of free iodine destroys the proportional variation of the color. Heat also increases the rate of color formation. By the color development in 0.4 N potassium iodide (5 cc. of 2 N KI in 25 cc.) in the presence of either acid or hot water, full color is developed immediately.

The color so formed is permanent for 24 hours or longer. The only interfering substances found are iron, which forms a colored iodide, copper, which precipitates out as the iodide in acid solution, ferricyanide, which gives a green color, and alcohol, which reduces the potassium iodoplatinate. The hotter the solution, the less actual color is obtained, but this color is still in proportion to the amount of potassium present. Exposure to bright sunlight through glass for 30 minutes liberates iodine, which obviously invalidates the determination. Indirect light or darkness does not cause oxidation of the iodine salt.

By application of the optimal conditions defined above to the production of the wine-red color, the amount of potassium present in a solution can be determined colorimetrically within the reading error of the instrument, which is considered as ± 4 per cent.

The potassium iodoplatinate is readily reduced in neutral solution by sodium thiosulfate. This reaction must take place

in a solution exactly neutral, as acid frees excess iodine and alkali suppresses the reaction. A final concentration of normal potassium iodide and heat are both used to insure a rapid formation of the iodine compound. Heat does not affect the actual titration with thiosulfate. Alcohol naturally must be avoided. Neutral salts such as potassium chloride have no effect. In the preliminary work the precipitate was separated and washed by centrifugalization. This procedure gave good results, but an occasional analysis in a series gave large variation. This led to an improved filtration method which we have adopted here.

Because of the color of the reduced salt in solution, which is a lemon-yellow, the iodine salt is a self-indicator. The end-point is taken as the place at which 1 drop of the thiosulfate changes the color of the solution from a reddish to a greenish yellow.

By titration of the iodoplatinate solution with 0.01 N sodium thiosulfate in a micro burette, 0.4 mg. potassium or more can be determined with an accuracy of ± 2 per cent.

The reduced salt tends to become reoxidized on standing in the air, especially in acid solution. Advantage is taken of this fact to check the titration value with a colorimetric comparison after complete oxidation. Quantitative transformation to potassium iodoplatinate is hastened by the addition of acid and a mild oxidizing agent, 0.2 per cent H_2O_2.

Both the ashing of the sample and the precipitation of the potassium chloroplatinate are carried out according to the well known Lindo-Gladding method for potassium. A few minor modifications have been introduced to make the procedure applicable to such small amounts of material as must be used. With materials such as amino acids which contain large amounts of nitrogenous substances in comparison to the potassium content, a preliminary ashing in glass with sulfuric acid and superoxol is recommended. Otherwise, 5 to 10 per cent of the potassium is lost. When a small amount of protein is present, as in pathological cerebrospinal fluids, the results may be 50 per cent low unless the protein is first removed. The potassium chloroplatinate is precipitated in the presence of redistilled alcohol and the wash liquids are saturated with the salt. In this way the last 0.01 mg. is precipitated.

Ammonia also forms a chloroplatinate which has the same

solubilities and properties as potassium chloroplatinate. Because of this, great care should be taken that all ammonia is expelled from the ash. A sulfuric acid ashing should be carried out on all samples just before the precipitation of the chloroplatinate to insure the volatilization of absorbed ammonia.

Analytical Procedure.

1. Reagents.

Trichloroacetic acid, 20 per cent.
Superoxol (30 per cent hydrogen peroxide) Merck, Blue Label.
Sulfuric acid, approximately 4 N.
Hydrochloric acid, approximately N.
Chloroplatinic acid, 10 per cent platinum.
Alcohol, redistilled over lime.
Alcohol, redistilled over lime, saturated with potassium chloroplatinate.
Potassium chloride, 10 per cent, saturated with potassium chloroplatinate.
Potassium iodide, 2 N, iodate-free, recrystallized from alcohol.
Sodium thiosulfate, 0.01 N. This is standardized against potassium iodate.
Hydrogen peroxide, 0.2 per cent (made up just before use).

2. Preparation of the Sample.

(*a*) *Blood Serum or Plasma.*—The determination is made on the protein-free filtrate. 1 volume of 20 per cent trichloroacetic acid is added to 1 volume of serum or plasma and diluted to 5 volumes. 5 cc. of the filtrate, equivalent to 1 cc. of the serum or plasma, are taken for the colorimetric determination. Twice this amount is taken for the titrimetric determination.

(*b*) *Whole Blood.*—1 volume of whole citrated blood is precipitated with 2 volumes of 20 per cent trichloroacetic acid and diluted to 5 volumes. 1 cc. of the human blood filtrate, which is equivalent to 0.25 or 0.35 mg. of potassium, is taken for the colorimetric procedure and twice the amount for the titrimetric determination. 2 to 5 cc. of dog blood filtrate are necessary to supply the correct amount of potassium.

3. Ashing of Sample.

(*a*) *Salt Solutions.*—These are ashed directly.

(*b*) *Serum, Plasma, and Cerebrospinal Fluid.*—An amount of the sample containing from 0.15 to 0.80 mg. of potassium is

placed in a Folin digestion tube. 0.6 cc. of 4 N sulfuric acid is added and the mixture is evaporated to a small volume. A drop or more of superoxol (30 per cent hydrogen peroxide) is added at intervals and the ashing is continued until a clear, colorless solution is obtained while sulfuric acid fumes are escaping. Care should be taken that all hydrogen peroxide has been dispelled before one continues. The sample is then transferred to a platinum crucible, evaporated to a small volume, and ashed according to the regular procedure described in (*c*).

(*c*) *Whole Blood, Urine, and Stool.*—An amount of the sample containing from 0.15 to 0.8 mg. of potassium is transferred to a platinum crucible. 4 drops of 4 N sulfuric acid solution are added. The sample is evaporated to dryness on a hot plate. The dish is heated carefully over a micro burner until all sulfuric acid fumes are given off and the ash becomes white. 1 drop of 4 N sulfuric acid is added and the sample re-ashed at red heat. With samples containing a larger percentage of potassium than that in blood, such as stool, it is often easier to ash more of the sample than is required for one determination. This is dissolved in a definite volume of water and aliquots are taken for the determination.

4. Precipitation of Potassium Chloroplatinate.

To the ash so prepared are added 1 drop of N hydrochloric acid and 0.30 cc. of platinic acid (10 per cent platinum). This is thoroughly mixed and 5 cc. of alcohol are added. After standing for 20 minutes, the precipitate is transferred to a micro filter (9). The filter is made by mounting a 1 inch funnel in a Witt filtering apparatus, which is essentially a suction flash with a ground glass removable top, so that the filtrate may be recovered in a small inner tube. A glass pearl is dropped into the funnel and a mat about $\frac{1}{32}$ of an inch thick of fine grained asbestos is put over the bead. This makes essentially a micro Caldwell crucible. The excess platinum is filtered off by suction and saved for recovery. The precipitate and the filter are then washed four or five times with alcohol saturated with potassium chloroplatinate. The contaminating salts, which are precipitated with the chloroplatinate in alcohol, are washed out with 3 or 4 washings of 10 per cent potassium chloride saturated with potassium chloroplatinate.

5. Quantitative Determination of Potassium Chloroplatinate.

The amount of potassium in the chloroplatinate so obtained can be determined in the following ways.

(*a*) *Colorimetric Determination.*—A 25 cc. volumetric flask is placed in a Witt filtering apparatus and the precipitate dissolved *in situ* by the repeated addition of small amounts of hot water which is first used to rinse the platinum crucible. This is followed by addition of 5 cc. of 2 N potassium iodide and 1 cc. of 1 N HCl. The mixture is cooled and made to the volume. The deep wine-red color can be compared against known standards at once.

TABLE I.

Recovery of Potassium from Known Solutions.

Authors' method.	Amount present.	Error.	Remarks.
mg.	*mg.*	*per cent*	
0.284	0.288	−1.3	Colorimetric procedure (centrifuged). Artificial blood salts.
0.289		+0.2	
0.288		±0	
0.267		−7.2	
0.292		+1.5	
0.581	0.576	−0.8	Titrimetric procedure (filtered). Artificial blood salts.
0.579		+0.5	
0.579		+0.5	
0.586		+1.6	
0.586		+1.6	
0.398	0.400	−0.4	Titrimetric procedure. K_2SO_4.
0.405		+1.0	

Colorimetric determination is convenient for 0.1 mg. to 4 mg. of potassium in the form of potassium chloroplatinate.

(*b*) *Titrimetric Procedure.*—The funnel containing the precipitate is removed from the Witt filtering apparatus, inverted, and the precipitate together with the asbestos returned to the original crucible by means of a small glass rod inserted in the stem of the funnel. The sides of the funnel are washed with 1 to 2 cc. of hot water and 1 cc. of 2 N potassium iodide. The mixture is heated almost to boiling for 3 to 5 minutes. It is not necessary to remove the asbestos. The solution is then titrated hot with 0.01 N sodium thiosulfate delivered from a micro burette calibrated to 0.01 cc.

TABLE II.

Potassium Determinations on Physiological Material.

Material.	Authors' method.	Gravimetric method.		Remarks.	Material.	Authors' method.	Gravimetric method.		Remarks.
	mg.	*mg. in aliquot*	*mg. per cent*			*mg.*	*mg. in aliquot*	*mg. per cent*	
Human	0.365	0.365	182	Titri-	Cow	0.464	0.447	223	Titri-
blood.	0.365			metric	blood	0.470			metric.
				proce-	—Con-	0.460			
	0.357			dure.	tin-	0.466			
	0.368				ued.	0.450			
	0.357					0.451			
	0.356					0.460			
	0.368					0.460			Colori-
	0.364					0.460			metric.
	0.375					0.436			
	0.375					0.458			
						0.441			
	0.375					0.436			
	0.372					0.448			
Cow	0.169	0.179		Colori-	Rat	0.909	0.940		Titri-
blood.	0.182			metric.	blood.	0.930			metric.
	0.174					0.930			
	0.178					0.941			
	0.176								
						0.465	0.470	235	Titri-
	0.182	0.179		Colori-		0.463			metric.
	0.181			metric.	Pig	0.512	0.501	250	Titri-
	0.174				blood.	0.520			metric.
	0.175					0.516			
	0.178					0.512			
	0.174								
	0.177				Dog	0.217	0.223		Colori-
					blood.	0.223			metric.
	0.087	0.089		Colori-		0.225			
	0.086			metric.					
	0.084					0.446	0.446		Titri-
	0.085					0.450			metric.
	0.086								

TABLE II—*Concluded.*

Material.	Authors' method.	Gravimetric method.		Remarks.	Material.	Authors' method.	Gravimetric method.		Remarks.
	mg.	*mg. in aliquot*	*mg. per cent*			*mg.*	*mg. in aliquot*	*mg. per cent*	
Dog blood—Continued.	0.425	0.446	22.3	Colorimetric following titrimetric above.	Urine ashings.	0.449	0.436		Titrimetric.
	0.444					0.449			
						0.449			
						0.433			
						0.426			
						0.411	0.407		Titrimetric.
						0.404			
Cow serum.	0.238	0.239		Colorimetric (centrifuged).		0.408			
	0.229					0.404			
	0.226					0.417			
	0.242					0.418			
						0.422			
	0.461	0.478	23.9	Titration (centrifuged).					
	0.454					0.745	0.768		Titrimetric.
						0.760			
Cow's milk.	0.662	0.650		Titrimetric.		0.762			
	0.640								

The end-point is a lemon-yellow color free from red. 1 cc. of thiosulfate is equivalent to 0.39 mg. of potassium. The titrimetric procedure is convenient for 0.4 to 1.0 mg. of potassium and potassium chloroplatinate.

(*c*) *Combination.*—The titrimetric result can be checked colorimetrically by reoxidation of the solution. 1 cc. of the normal hydrochloric acid solution and 0.10 cc. of a 0.2 per cent hydrogen peroxide solution are added to the mixture. This is allowed to stand exposed to the air from 30 minutes to 1 hour. The solution is made up to 50 cc. and compared against a suitable colorimetric standard.

The results obtained by this procedure are given in Tables I and II.

Determinations were made in triplicate or quadruplicate with good agreement. It is needless to say that known solutions of potassium sulfate were determined correctly; that a mixture of

salts of the same composition as the blood and salts of known purity gave theoretical values for potassium; and that potassium added to various unknowns was recovered. In addition, varying amounts of material yielded the same percentage analysis for potassium. All results with the present method were checked by gravimetric determination according to the Lindo-Gladding method. The results for blood, serum, milk, and urine were also checked by this method.

SUMMARY.

A procedure is outlined for the determination of 0.1 mg. or more of potassium. Potassium chloroplatinate is precipitated and converted to iodoplatinate. Quantitative determination is made either by colorimetric comparison with known standards, or by titration with sodium thiosulfate. By this method 0.1 mg. can be determined, ±4 per cent, and 0.4 mg., ±2 per cent.

BIBLIOGRAPHY.

1. Official and tentative methods of analysis of the Association of Official Agricultural Chemists, Washington, 1920, 12.
2. Tisdall, F. F., and Kramer, B., Methods for the direct quantitative determination of sodium, potassium, calcium, and magnesium in urine and stools, *J. Biol. Chem.*, 1921, xlviii, 1. Kramer, B., and Tisdall, F. F., The direct quantitative determination of sodium, potassium, calcium, and magnesium in small amounts of blood, *J. Biol. Chem.*, 1921, xlviii, 223.
3. Kerr, S. E., Studies on the inorganic composition of blood. I. The effect of hemorrhage on the inorganic composition of serum and corpuscles, *J. Biol. Chem.*, 1926, lxvii, 689, (694).
4. Kramer, B., and Gittleman., I., The gasometric determination of potassium, *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 241.
5. Yoshimatsu, S., A colorimetric method for determination of potassium with 0.2 cc. of blood, *Tohoku J. Exp. Med.*, 1926-27, viii, 174.
6. Bowser, L. T., Some features of the cobalti-nitrite method for potassium, *U. S. Dept. Agric., Bureau Chemistry, Bull. 152;* 1912, 45.
7. Cameron, F. K., and Failyer, G. H., The determination of small amounts of potassium in aqueous solutions, *J. Am. Chem. Soc.*, 1903, xxv, 1063.
8. Peterson, H., Volumetrische Bestimmung des Goldes und Platins, *Z. anorg. Chem.*, 1899, xix, 59.
9. Shohl, A. T., Apparatus for micro-filtration, *J. Am. Chem. Soc.*, 1928, l, 417.

THE RESORPTION OF COPPER AND FERROCYANIDE IONS BY COAGULATED PROTEINS.*

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It has been known for a long time that proteins are precipitated by, and precipitate, copper and other heavy metals; likewise, many acids behave in a similar way toward soluble proteins. Harnack (1) believed he showed a definite composition of the copper egg albumin precipitate. Galeotti (2), on the other hand, thought he found that copper is distributed between the protein precipitate and the solution according to the phase rule. Many other workers have come to varying conclusions concerning the nature of the compounds of copper and other heavy metals with proteins. Mathews (3) states that ions form salts with proteins, but does recognize that copper and other heavy metals may enter into combination with the amino group of the protein molecule. Loeb (4) has shown that the combination between gelatin and hydroferrocyanic acid is of the nature of a salt.

In view of the recent attention which has been given to the presence of small amounts of certain elements in the animal organism, it has been of interest to study the concentration of copper and of hydroferrocyanic acid on coagulated protein from very dilute solutions.

Methods.

Coagulated protein was used in these experiments because this made possible the separation of the protein for analysis from the solution with as little manipulation as possible. It was necessary only to filter off the coagulated protein and weigh the fraction collected. Had uncoagulated protein been used, it would have been necessary to precipitate the protein by some method which probably would have disturbed the equilibrium between the

* A preliminary report of this work was read before the American Society of Biological Chemists at Ann Arbor, April 13, 1928.

copper in solution and the copper which had combined with the protein.

Coagulated egg albumin and coagulated edestin were used in these experiments. In order to save space, this discussion will be confined to the experiments with egg albumin. The coagulated egg albumin was prepared from crystallized egg albumin. It was thoroughly washed to remove salts and other soluble substances, and was found to be entirely free from copper but contained a very faint trace of iron.

In every case 1 gm. of coagulated protein was used. In most cases the protein was suspended in 2810 cc. of solution which contained 10 mg. of copper or 10 mg. of iron in the form of potassium ferrocyanide. In a few experiments, the volume of the solution was 5000 cc. which contained 5 mg. of copper. 1 gm. of protein was used in each of these experiments. In certain cases, the volume of solution was very slightly increased by the addition of acid or alkali in adjusting the pH of the solution. In many of the experiments with copper, no attempt was made to regulate the hydrogen ion concentration of the solution, which doubtless accounts for the rather wide fluctuations in the results obtained. In some of the later experiments, the pH was regulated and varied from pH of 3.4 to 6.9. Some experiments were carried out at pH lower than 3.4, but since the protein took up no copper, they are not reported here in detail. No experiments were carried out at pH above 7. It was feared the copper might be precipitated as the hydroxide above this point.

In the experiments with potassium ferrocyanide, the pH was varied between 3 and 6.3. Experiments with ferrocyanide were not attempted at higher acidity than that represented by pH 3, because even at this level there is some evidence of the decomposition of ferrocyanide. The protein was allowed to remain in solution from 2 to 6 days, but all evidence seemed to indicate that equilibrium had been reached in 2 days.

In filtering the protein, no effort was made to collect all of the protein, but 75 to 90 per cent of it was collected on an ash-free filter paper and washed with a very little water, drained with the aid of reduced pressure, and dried to constant weight. From the determination of copper or iron in this portion of the protein, the amount in 1 gm. was calculated.

The copper was determined by a method which has been previously reported by the writer (5), and the iron, at first, by the method of Elvehjem and Hart (6) and later by the method of Kennedy (7).

Results.

In order to study adequately the results of these experiments, it seems necessary to pay careful attention, not only to the actual amount of copper and of iron combined with 1 gm. of protein, but also to the ratio between the amount of copper or iron combined with 1 gm. of the coagulated protein and the amount found in an equal amount of the solution.

The results of the first series of experiments showed that the average ratio between copper in protein to that found in an equal amount of solution was 2974 for eight experiments. An average of 5.05 mg. of copper, or 50.0 per cent of the total copper present, was taken up by the protein. The maximum variation in this series of experiments was quite large, being 1.72 mg., or 17.2 per cent of the total.

The results obtained in this series of experiments raise the following questions. (1) Will coagulated protein take up only half of the copper from solutions under conditions such as were maintained in this series of experiments? (2) What are the factors which cause the quite large variations in the amount of copper taken up by protein? (3) Is this variation produced by variations in hydrogen ion concentration or is it the result of some surface relation between the protein and the solution?

In order to obtain an answer to the first of these questions, the volume of the solution was increased to 5010 cc. in two experiments. It was found that the actual amount of copper taken up was not much different from that in the experiment in which the volume was only 2810 cc. The ratio of concentration of copper in protein to that in the solution was markedly increased, being about 6000. These experiments indicate that dilution does not diminish to any extent the amount of copper which will be taken up by protein, providing the amount of copper in the system remains unchanged. Two other experiments were carried out in which the volume was 5005 cc. and only 5 mg. of copper were present. In these experiments, the actual amount of copper taken up was

only slightly less than in those experiments in which the volume was only 2800 cc. and the amount of copper was 10 mg. The per cent of the total copper taken up, however, was much greater, being 95.8 and 81.8. In one case the ratio between copper in the protein and the copper in the solution rose to the very high value of 114,319. This, it is believed, shows a very high affinity of coagulated protein for copper, at least until about 5 mg. of copper have

TABLE I.

1 gm. of coagulated egg albumin brought to equilibrium with 2800 cc. of distilled water, 10 cc. of a solution of copper sulfate which contained 10 mg. of copper, and enough hydrochloric acid or sodium bicarbonate to bring the solution to the desired pH.

Date.	pH	Cu in 1 gm. protein.	Cu in solution.	Total Cu in protein.	Cu in solution.
1927		*mg.*	*mg.*	*per cent*	*p. p. m.*
July 8–14	3.4	0.302	9.698	3.02	3.44
" 8–14	3.4	0.302	9.698	3.02	3.44
" 8–14	3.5	0.436	9.564	4.36	3.39
" 8–14	3.5	0.455	9.545	4.55	3.38
June 27–30	4.3	1.39	8.61	13.9	3.06
" 27–30	4.3	1.77	8.23	17.7	2.92
July 8–14	5.2	2.55	7.45	25.5	2.651
" 8–14	5.2	2.54	7.46	25.4	2.654
" 8–14	5.2	2.75	7.25	27.5	2.58
" 8–14	5.6	3.22	6.78	32.2	2.41
" 21–27	6.9	5.87	4.13	58.7	1.47
" 21–27	6.9	5.73	4.27	57.3	1.52
" 21–27	6.9	6.00	4.00	60.0	1.42

been taken up by each gm. of the protein. These experiments show that dilution does not influence the taking up of copper by protein in any important way, providing the total amount of copper in the mixture is not reduced below 5 mg.

In these more dilute solutions, there is considerable variation in the amount of copper taken up by the protein. The very small amount of copper left in solution makes the ratio very high and

also increases the variation in these ratios because experimental errors are multiplied by such large figures. It appears certain, however, that this fluctuation is not all due to experimental error.

The study of these fluctuations in the amount of copper taken up by coagulated protein dealt entirely with the effect of varying the

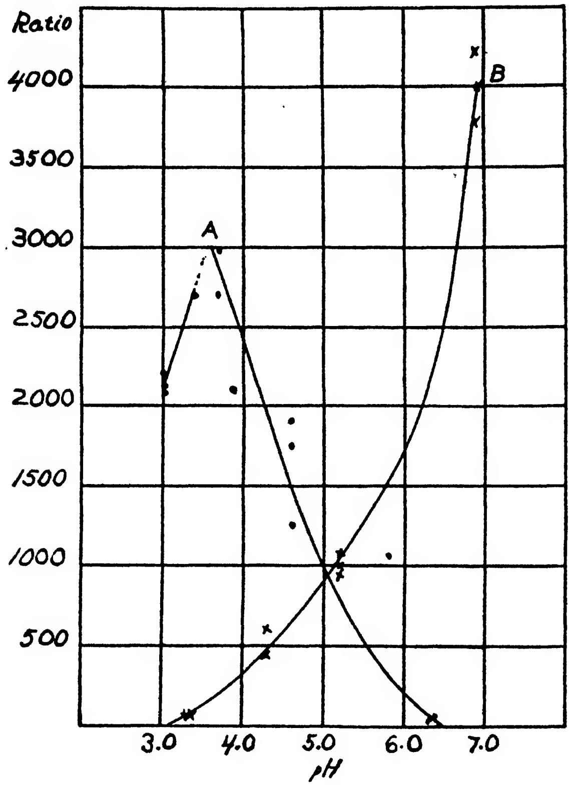

Fig. 1. Showing the effect of pH upon the distribution of ions between coagulated egg albumin and water. *A*, ferrocyanide; *B*, copper.

hydrogen ion concentration of the solution. In these experiments, 2800 cc. of water, 10 cc. of a solution containing 10 mg. of copper in the form of the sulfate, and enough of a solution of hydrochloric acid or sodium bicarbonate (see Table I for exact volume) to obtain the desired pH were allowed to come to equilibrium with 1 gm. doses of coagulated egg albumin. Solutions of pH from 2.5 to

6.9 were used in these studies. It was found that the protein took up no copper at a pH of 2.5 and virtually none at pH 3, so that Table I includes results only from pH 3.4 to 6.9. No experiments were carried out above pH 6.9 on account of the possible danger of the precipitation of copper at higher pH values. The ratio of copper combined with the protein to that in an equal amount of the solution varied from an average of 3995 at a pH of 6.9 to 87.5 at a pH of 3.4 and, of course, to 0 at a pH of 2.5. There was found in the protein an average of 58.3 per cent of the total copper when the pH was 6.9 and 3.02 per cent at a pH of 3.4. The variations between these extremes are shown in Table I and in Fig. 1. It will be noted that the curve in Fig. 1 resembles a titration curve to a certain extent. The number of determinations reported is not sufficient to enable one to say that the form of the curve is correct, but it is felt that it shows in an approximate manner the variation of the amount of copper which will combine with coagulated protein at varying hydrogen ion concentrations.

In view of this variation of the combination of copper with protein at different hydrogen ion concentrations, it seemed worth while to investigate the combination of a negative ion with coagulated egg albumin at varying hydrogen ion concentrations. As mentioned above, ferrocyanide was used for this purpose. The experiments with ferrocyanide were carried out exactly the same as in the preceding series of experiments, except that potassium ferrocyanide which contained 10 mg. of iron was used in place of copper. Table II and Fig. 1 give the variation in the combination of ferrocyanide with coagulated protein at different pH values. The results are calculated in mg. of iron combined with 1 gm. of protein and the ratio between the amount of iron in 1 gm. of protein to the iron in an equal amount of solution. It will be noticed that the protein contained an average of 51.04 per cent of the total iron, and the ratio was 2908 at a pH of 3.7, while at pH of 6.3 an average of only 1.72 per cent of all the iron was found in the protein, and the ratio was 45.7. There was a definite decrease in the amount of iron combined with the protein in solutions more acid than pH of 3.7. It is believed that this is to be accounted for by the decomposition of hydroferrocyanic acid in acid solution. In fact, the work with ferrocyanide was made unsatisfactory by this tendency of the ferrocyanide. These results, although very few

in number, do show that coagulated protein combines with ferrocyanide and presumably with other anions to a greater extent in more acid solutions.

The type of combination between the coagulated protein and the copper and ferrocyanide appears to be the same as with uncoagulated protein. If this combination is of the nature of adsorption, it is surprising that the results are so constant at a definite pH, for it cannot be supposed that any two doses of dried coagulated egg albumin or edestin would present the same surfaces to the solutions. It appears, therefore, that the combination between coagulated protein and the copper and the ferrocyanide is a chemical one in the same sense that uncoagulated protein combines with acidic or basic radicals.

TABLE II.

1 gm. of coagulated egg albumin brought to equilibrium with 2800 cc. of distilled water, 10 cc. of a solution of potassium ferrocyanide which contained 10 mg. of iron, and enough hydrochloric acid or sodium bicarbonate to bring the solution to the desired pH.

Date.	pH	Fe in 1 gm. protein.	Fe in solution.	Total Fe in protein.	Fe in solution.
1927		*mg.*	*mg.*	*per cent*	*p. p. m.*
July 31–Aug. 4	3.0	4.28	5.72	42.8	2.03
" 31– " 4	3.0	4.38	5.62	43.8	1.99
" 31– " 4	3.0	4.31	5.69	43.1	2.01
" 31– " 4	3.0	4.24	5.76	42.4	2.04
" 31– " 4	3.4	4.84	5.16	48.4	1.832
" 31– " 4	3.4	4.865	5.135	48.65	1.823
Aug. 15–17	3.7	5.308	4.692	53.08	1.77
" 15–17	3.7	4.90	5.10	49.00	1.81
" 15–17	3.9	4.25	5.75	42.5	2.04
" 15–17	4.6	4.05	5.95	40.5	2.11
" 15–17	4.6	3.08	6.92	30.8	2.45
" 15–17	4.6	3.85	6.15	38.5	2.18
" 7–11	5.8	2.74	7.26	27.4	2.544
" 7–11	6.3	0.165	9.835	1.65	3.93
" 7–11	6.3	0.190	9.81	1.90	3.92

SUMMARY.

It is shown that coagulated proteins take up copper from dilute solutions, and if the solutions are dilute enough, almost all of the copper may be taken out of solution.

The amount of copper taken up by coagulated egg albumin increases with the pH at least up to the pH of 6.9.

Preliminary studies indicate that the coagulated protein takes up the acid radical ferrocyanide in acid solution and the amount of ferrocyanide combined increases as the pH decreases to the pH of 3.7.

BIBLIOGRAPHY.

1. Harnack, E., *Z. physiol. Chem.*, 1881, v, 198.
2. Galeotti, G., *Z. physiol. Chem.*, 1903, xl, 492.
3. Mathews, A. P., Physiological chemistry. A text-book and manual for students, New York, 4th edition, 1925.
4. Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 2nd edition, 1924.
5. Hendrix, B. M., *J. Biol. Chem.*, 1927, lxxiv, p. vii.
6. Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1926, lxvii, 43.
7. Kennedy, R. P., *J. Biol. Chem.*, 1927, lxxiv, 385.

THE EFFECT OF SODIUM CITRATE, ACETATE, AND LACTATE ON THE ULTRAFILTRABILITY OF SERUM CALCIUM.

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The toxicity of sodium citrate was described by Metcherlich (1) in 1845, but little was known of its mode of action in the animal organism until the studies of Sabatini (2) and of Loeb (3) in 1901. Loeb (3) and Zoethout (4) attributed the tetanic contractions produced in muscles immersed in sodium citrate or oxalate solutions to the formation of insoluble calcium salts of citrate and oxalate. This explanation of the toxicity of sodium citrate has been questioned by Gros (5) who demonstated that sodium tartrate does not produce convulsions when injected intravenously, although calcium tartrate is less soluble than calcium citrate. Sabatini (6) found that the quantity of the serum calcium does not alter after sodium citrate injection, and he therefore suggested that sodium citrate invokes a physical or chemical change in the serum calcium so that it becomes no longer available as a regulator of muscular contraction and relaxation. He concludes that since calcium citrate is soluble, it must be that it forms an unionized compound.

With the exception of Gross (7), who injected dogs subcutaneously with dilute solutions of sodium citrate, all modern investigators agree that this salt is capable of producing tetanic muscular contractions when introduced in the living animal body (8–14).

The following investigation suggested itself after an accidental observation. In the course of some dietary experiments with young rabbits, sodium citrate was added to their milk rations to promote digestibility, as recommended by Hart (15). On several occasions rabbits were found dead in their cages and even necropsy did not reveal the cause of death. In one instance, however, a small rabbit was observed to be in a state of tonic and clonic convulsions soon after partaking of the milk diet. We

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suspected that the ingested sodium citrate found its way into the circulation and formed an undissociable calcium compound, thereby causing convulsions. Blood was obtained by cardiac puncture and the serum calcium was found to be within the normal limits for rabbits of that age and size, and apparently in agreement with the observation of Sabatini. Part of the serum was ultrafiltered in a collodion bag[1] at a pressure of 120 to 150 mm. of mercury. The calcium concentration of the filtrate was determined and compared with the ultrafiltrate of normal rabbit serum. It was found that, whereas only 45 to 57 per cent of the serum calcium of normal rabbits is ultrafiltrable, nearly 100 per cent of the serum calcium of the citrate-fed rabbit passed into the filtrate.

The following experiments were planned to confirm this observation.

I. Effect of Intravenous Injection of Sodium Citrate on Ultrafiltrability of Serum Calcium.

Preliminary to injection, blood was obtained from large adult rabbits by cardiac puncture. Then the desired solution was injected into the marginal ear vein of the animal and as soon as convulsions appeared blood was again obtained by cardiac puncture. The blood was collected in dry, clean centrifuge tubes, allowed to clot, centrifuged for about 15 minutes, and the clear serum separated. Part of the serum was used for ultrafiltration. The ultrafiltrate, as well as the undialyzed serum, was analyzed for calcium by the method of Kramer and Tisdall (16).

In the first experiments 1 cc. of a 30 per cent solution of sodium citrate was employed and before the needle was withdrawn from the vein, tonic and clonic convulsions occurred. Within a minute, the animals were in opisthotonos, and died of respiratory and circulatory failure. On rare occasions animals were revived by the application of amyl nitrite to their nostrils as suggested by Normet (9).

When sodium citrate was injected in more dilute solutions so

[1] The bags were made of 6 per cent du Pont parlodion dissolved in 25 cc. of absolute alcohol and 75 cc. of ether. About three coats of the solution were sufficient to make a bag which would withold the desired pressure and render the filtrate protein-free.

that the rate of injection was slower, a greater quantity of this salt was necessary to produce convulsions and the effect did not appear until 3 to 4 minutes after injection. The effect on the ultrafiltrability of serum calcium was, however, the same as when

TABLE I.

Effect of Sodium Citrate on Ultrafiltrability of Serum Calcium of Rabbits.

Date.	Rabbit No.	Amount injected.	Concentration.	Before injection.		Per cent ultrafiltrable.	After injection.		Per cent ultrafiltrable.	Remarks.
				Ca in serum.	Ca in ultrafiltrate.		Ca in serum.	Ca in ultrafiltrate.		
1926		*cc.*	*per cent*	*mg. per cent*	*mg. per cent*		*mg. per cent*	*mg. per cent*		
Feb. 1	1	2.0	30	14.6			15.2			Convulsions within 1 min.
" 1	2	1.0	30	16.8	7.3	45	14.3	12.0	86	" "
" 5	3	11.0	10	13.9	8.3	57	14.4	13.2	97	Convulsions within 3 min.

TABLE II.

Effect of Addition of Sodium Citrate to Serum in Vitro on Ultrafiltrability of Serum Calcium.

Source of serum.	Amount of serum.	Amount of 30 per cent citrate added.	Before addition.		Per cent ultrafiltrable.	After addition.		Per cent ultrafiltrable.
			Ca in serum.	Ca in ultrafiltrate.		Ca in serum.	Ca in ultrafiltrate.	
	cc.	*cc.*	*mg. per cent*	*mg. per cent*		*mg. per cent*	*mg. per cent*	
Sheep.	15.0	1.0	10.7	4.9	45	10.6	10.4	99
"	15.0	1.0	10.0	6.4	64	10.0	8.9	89
"	15.0	1.0	9.6	4.2	43	9.6	9.6	100
Human.	15.0	1.0	10.7	4.5	42	10.7	8.9	84
"	15.0	1.0	10.7	4.8	45	10.7	10.5	98
Cow.	10.0	0.5	10.4	5.0	48	10.4	10.2	99
"	10.0	0.5	10.4	5.0	48	10.4	10.0	98

a more concentrated solution was employed. Table I shows that whereas only 45 to 57 per cent of the serum calcium of normal rabbits is ultrafiltrable, the serum calcium of rabbits injected with sodium citrate is 86 to 97 per cent ultrafiltrable.

II. Effect of Sodium Citrate on Ultrafiltrability of Serum Calcium in Vitro.

That the effect of sodium citrate on the state of serum calcium is the same whether added *in vivo* or *in vitro* may be seen in Table II. It shows that similar results are obtained *in vitro* with the serum of sheep, cow, and man.

III. Effect of pH on Ultrafiltrability of Serum Calcium in Vitro.

Although the effect of sodium citrate on the diffusibility of calcium is the same as if acid were added to serum, as can be inferred from Table III, where the pH was adjusted to 5.1 with

TABLE III.

Effect of pH on Ultrafiltrability of Serum Calcium.

Source of serum.	Amount of serum.	Amount of 30 per cent sodium citrate added.	pH	Ca in serum.	Ca in ultrafiltrate.	Per cent ultrafiltrable.
	cc.	*cc.*		*mg. per cent*	*mg. per cent*	
Cow.	10.0	0.0	7.2	10.4	5.0	48
"	10.0	0.5	8.3	10.4	10.2	99
"	10.0	0.5	8.2	10.4	10.0	98
"	10.0	0.5	5.9*	10.4	10.5	100
"	10.0	0.5	5.1*	10.4	10.6	100
"	10.0	0.0	5.1*	10.4	10.5	100

* Serum rendered acid by addition of HCl.

HCl without the addition of sodium citrate, yet the citrate effect is not due to acid. The citrate effect is evident when the serum is made more acid, equal to, or more alkaline than blood serum. If the change in reaction were the controlling factor, a decrease rather than an increase in the ultrafiltrable calcium should be expected, since the addition of sodium citrate renders the solution more alkaline.

IV. Effects of Sodium Acetate and Sodium Lactate.

The effects of sodium acetate and lactate were studied *in vitro* and *in vivo*. At first 30 per cent solutions of these salts were used even in correspondingly larger amounts than of the sodium citrate

TABLE IV.

Effect of Injection of Sodium Citrate, Acetate, and Lactate into Rabbits.

Date.	Rabbit No.	Amount of 30 per cent solution injected.	Before injection. Ca in serum.	Before injection. Ca in ultrafiltrate.	Per cent ultrafiltrable.	After injection. Ca in serum.	After injection. Ca in ultrafiltrate.	Per cent ultrafiltrable.	Remarks.
1926		*cc.*	*mg. per cent*	*mg. per cent*		*mg. per cent*	*mg. per cent*		
June 17	4	6.0 Na acetate.	14.2	7.8	55	14.0	7.8	55	No convulsions.
" 18	4	1.0 " citrate.				12.0	12.0	100	Convulsions in 1 min.
Stoichiometric amounts.									
1927									
Aug. 11	5	10.0 Na citrate.	15.8	8.5	53	15.8	16.0	100	Convulsions in 1 min.
	6	10.0 " acetate.	14.0	6.8	48	15.5	8.1	52	No convulsions.
	7	10.0 " lactate.	14.5	6.6	45	14.5	9.9	61	" "
	8	2.0 " citrate.	14.0	10.0	70	12.8	13.0	100	Convulsions at once.
	9	2.0 " acetate.	15.6	6.7	43	14.6	7.0	48	No convulsions.
	10	5.0 " lactate.	15.6	7.8	50	15.6	10.5	67	" "

TABLE V.

Effect of Addition of 0.2 Cc. of Stoichiometric Solutions of Sodium Citrate, Acetate, and Lactate on Ultrafiltrability of Serum Calcium (Sheep).

Amount of serum.	Solution added.	Ca in serum.	Ca in ultrafiltrate.	Per cent ultrafiltrable.
cc.		*mg. per cent*	*mg. per cent*	
15	None.	11.6	4.6	40
15	Na citrate.	11.6	9.9	85
15	" acetate.	11.6	4.9	42
15	" "	11.6	4.9	42
15	" lactate.	11.6	5.8	50
15	" "	11.6	5.7	49
15	" "	11.6	5.9	51

but no convulsions resulted nor was the ultrafiltrability increased to the extent obtained with sodium citrate. Later these experiments were repeated with stoichiometric concentrations of the three salts with similar results.

It is evident from Tables IV and V that, whereas sodium citrate evoked convulsions within 1 minute after injection, no convulsions occurred when sodium acetate or lactate was introduced; and that whereas the serum calcium is nearly 100 per cent diffusible when sodium citrate is present, the diffusibility is only slightly increased by the introduction of sodium lactate and not at all increased by sodium acetate.

DISCUSSION.

In 1911, Rona and Takahashi (17), employing the method of compensation dialysis, found that serum calcium exists in a diffusible and a non-diffusible form. The latter fraction, they suggested, was most likely bound to serum protein. Using the method of ultrafiltration, Cushny (18), Neuhausen and Pincus (19), von Meysenbug, Pappenheimer, Zucker, and Murray (20), Moritz (21), and Updegraff, Greenberg, and Clark (22) found that 45 to 50 per cent of the serum calcium is non-diffusible. Cameron and Moorhouse (23) proposed that the non-diffusible calcium is bound to some organic substance which may be the parathyroid hormone or is regulated by it. Salvesen and Linder (24) in their studies on nephritis noticed the parallelism between the reduction in serum calcium and protein. They also suggested that the non-diffusible calcium of the serum exists in a protein-like combination. Their indirect evidence has been elaborated by Loeb (25) and Marrack and Thacker (26). The former ascribes the diffusible and non-diffusible calcium to the "Donnan effect" and to a lesser extent to the formation of a complex calcium-protein compound. The latter investigators minimize the rôle of the "Donnan equilibrium" and explain their results mainly on the calcium-protein theory.

It is not the purpose of this paper to enter into a discussion regarding the state of the non-diffusible fraction of the serum calcium. It is desirous, however, to point out that our results in respect to the diffusible and non-diffusible portions of normal serum calcium agree well with those reported in the literature;

and in addition, to indicate the specific effect of sodium citrate on the non-diffusible fraction whether introduced *in vivo* or *in vitro.*

The effect of citrates on biologic systems has been known for nearly a century, but more recently their effect on systems containing calcium has been studied more systematically. Hastings, Murray, and Sendroy (27) demonstrated changes in the solubility of calcium phosphate and carbonate when sodium citrate was present; and Shipley, Kramer, and Howland (28) were unable to obtain calcification *in vitro* even with a Ca $\times$ P product of 60 if the calcium was present as calcium citrate.

As to the mechanism of the citrate effect the hypothesis of Sabatini that citrate ions combine with calcium to form a soluble, unionizable compound seems plausible. That calcium, although still soluble, is removed from solution when citrate ions are added was demonstrated by Shear and Kramer (29) in this laboratory. They studied the ionic interaction between calcium and citrate by means of conductivity titrations and found that the addition of a solution of sodium citrate to one of calcium chloride resulted in a binding of the calcium ions by the citrate. In subsequent experiments, performed at 38° Kramer, Shear, and Resnikoff (30) found that lactate and acetate behave normally towards calcium ions, *i.e.*, in the same manner as chlorides, while citrate ions show the effect noted above, *i.e.*, a diminished ionic activity as indicated by a decrease in conductivity.

Sabatini explained the convulsive seizures as due to the inactivation of calcium which is necessary for muscular contraction and relaxation. Normet attributes the shock phenomenon to the exciting action of sodium citrate on the sympathetic nervous system. Citric acid is absorbed by positive ions with the liberation of sodium, which is thus ready to take violently, from their places, the metallic ions of the tissues. This explanation of the mode of action of sodium citrate is supported neither by experimental evidence nor by theoretical speculation. Our ultrafiltration experiments and the observations of Shear and Kramer would seem to support Sabatini's hypothesis; namely, that the citrate ions combine with calcium to form an unionized compound and thus rob the organism of its active calcium necessary for muscular and nervous stimulation and inhibition.

The beneficial effects of sodium citrate in peripheral hemorrhages have been attributed, by Neuhof and Hirshfeld (31), to a decrease in clotting time. This, however, has been questioned by Joannides (11). It is more plausible to explain the decrease in bleeding time as due to vasoconstriction as suggested by Goia and Petri (32) and by Normet, not because of the liberation of sodium ions but rather to the inactivation of calcium by citrate ions and thereby causing contraction of the muscular coats of the blood vessels. This seems plausible because of the resuscitating effects of vasodilators such as amyl nitrite in animals with sodium citrate convulsions.

From a therapeutic standpoint, it seems that dilute solutions of sodium citrate when injected intravenously cause vasoconstriction and prevent bleeding; while concentrated solutions cause convulsions and death. Joannides reports shock in a patient treated with intravenous sodium citrate, who subsequently recovered by the administration of calcium chloride; and Kionka (33) reports an immediate death in a patient so treated.

SUMMARY AND CONCLUSIONS.

1. Sodium citrate when injected intravenously into rabbits in 30 per cent solution produces almost instantaneous convulsions and death.
2. The serum calcium of such rabbits becomes nearly 100 per cent ultrafiltrable as compared to serum of normal rabbits which is only 45 to 47 per cent ultrafiltrable.
3. Sodium citrate when added to serum *in vitro* renders the calcium nearly 100 per cent ultrafiltrable.
4. The injection of sodium lactate or acetate into rabbits does not produce convulsions, nor do these salts increase the ultrafiltrability of serum calcium *in vitro* or *in vivo.*
5. These effects may be due to the formation of a soluble, unionizable calcium citrate compound.

We are grateful to Dr. Benjamin Kramer for his kindly interest and criticisms and to Miss Elsa R. Orent for invaluable technical assistance.

BIBLIOGRAPHY.

1. Metcherlich, C. G., Dissertation, Berlin, 1845, quoted by Salant (12).
2. Sabatini, L., *Arch. ital. biol.*, 1901, xxxvi, 417.
3. Loeb, J., *Am. J. Physiol.*, 1901, v, 362.
4. Zoethout, W. D., *Am. J. Physiol.*, 1902, vii, 199, 320.
5. Gros, O., *Arch. Exp. Path. u. Pharmakol.*, 1913, lxxi, 395.
6. Sabatini, L., *J. physiol. int.*, 1901, xxx, 2.
7. Gross, E. G., *J. Biol. Chem.*, 1923, lv, 729.
8. Gates, F. L., and Meltzer, S. J., *J. Exp. Med.*, 1916, xxiii, 655.
9. Normet, R., *Bull. Acad. méd.*, 1924, xcii, 1306.
10. Panisset, L., and Verge, I., *Comp. rend. Soc. biol.*, 1922, lxxxvii, 224.
11. Joannides, M., *Arch. Int. Med.*, 1926, xxxvii, 248.
12. Salant, W., and Wise, L. E., *J. Biol. Chem.*, 1916–17, xxviii, 27.
13. Salant, W., and Swanson, A. M., *J. Pharmacol and Exp. Therap.*, 1918, xi, 133.
14. Salant, W., and Hecht, S., *Am. J. Physiol.*, 1915, xxxvi, 126.
15. Hart, E. B., Steenbock, H., Elvehjem, C. A., and Waddell, J., *J. Biol. Chem.*, 1925, lxv, 67.
16. Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlviii, 223.
17. Rona, P., and Takahashi, D., *Biochem. Z.*, 1911, xxxi, 336.
18. Cushny, A. R., *J. Physiol.*, 1919–20, liii, 391.
19. Neuhausen, B. S., and Pincus, J. B., *J. Biol. Chem.*, 1923, lvii, 99.
20. von Meysenbug, L., Pappenheimer, A. M., Zucker, T. F., and Murray, M. F., *J. Biol. Chem.*, 1921, xlvii, 529.
21. Moritz, A.R., *J. Biol. Chem.*, 1925, lxiv, 81.
22. Updegraff, H., Greenberg, D. M., and Clark, G. W., *J. Biol. Chem.*, 1926–27, lxxi, 87.
23. Cameron, A. T., and Moorhouse, V. H. K., *J. Biol. Chem.*, 1925, lxiii, 687.
24. Salvesen, H. A., and Linder, G. C., *J. Biol. Chem.*, 1923–24, lviii, 635.
25. Loeb, R. F., *J. Gen. Physiol.*, 1925, viii, 451.
26. Marrack, J., and Thacker, G., *Biochem. J.*, 1920, xx, 580.
27. Hastings, A. B., Murray, C. D., and Sendroy, J., Jr., *J. Biol. Chem.*, 1926–27, lxxi, 723.
28. Shipley, P. G., Kramer, B., and Howland, J., *Biochem. J.*, 1926, xx, 379.
29. Shear, M. J., and Kramer, B., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 624; *J. Biol. Chem.*, 1928, in press.
30. Kramer, B., Shear, M. J., and Resnikoff, L., unpublished data.
31. Neuhof, H., and Hirshfeld, S., *Ann. Surg.*, 1922, lxxvi, 1.
32. Goia, I., and Petri, I., *Paris méd.*, 1927, lxiii, 137.
33. Kionka H., quoted by Kobert, R., in Lehrbuch der Intoxikationen, Stuttgart, 2nd edition, 1896, i.

FACTORS AFFECTING THE ACCURACY OF THE QUANTITATIVE DETERMINATION OF VITAMIN A.*

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The quantitative determination of vitamin A by means of the rat growth method has in recent years become a matter of such importance as to repay extended study of the several factors influencing the accuracy of the results obtainable by the method as now developed.

The purpose of the present paper is to summarize, as concisely as possible, the findings of a number of experiments planned with special reference to this problem together with the results of a critical examination of the data of some hundreds of determinations of vitamin A which have been made in this laboratory in the course of investigations upon the distribution of this substance in nature and its behavior under various conditions. Except as otherwise explained, the general procedure in the experiments from which the data here discussed were drawn, has been that described by Sherman and Munsell.[1]

Selection of Experimental Animals.

All of the experiments here considered were made with young albino rats, 28 to 29 days old at the beginning of the "depletion period," *i.e.* when placed upon the vitamin A-free basal diet for depletion of the bodily store of this vitamin; and all were drawn from families which have been for many generations under observation in this laboratory and are of strictly known nutritional

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[1] Sherman, H. C., and Munsell, H. E., *J. Am. Chem. Soc.*, 1925, xlvii, 1639.

history. As most of these families are being used in nutrition experiments continued through successive generations, our practise has been not to "breed from the best" but to use, as breeders, animals which are strictly representative of the average. Hence our animals are, at present, not so large as those of many laboratories. Another influence probably acting in the same direction is the fact that, in the experiments here described, we did not artificially reduce the number of young in any litter, nor restrict the number of times of breeding, but allowed each female to produce and rear as many young as she could. Under these conditions, most of our young rats weigh between 35 and 55 gm. when 28 days old, and in general only animals within these limits of size are used in experiments such as those with which the present paper deals.

Considerable numbers of experiments have been made with young from each of the two diets hitherto most largely used in this laboratory, *viz.* Diet A (Laboratory No. 16) and Diet B (Laboratory No. 13). Diet A consists of one-sixth dried whole milk and five-sixths ground whole wheat; Diet B, of one-third dried whole milk and two-thirds ground whole wheat. In each case sodium chloride is added in the proportion of 2 per cent of the weight of the wheat in the food mixture, and distilled water is furnished *ad libitum.* The young from families on the diet containing the larger proportion of milk are found to have larger bodily stores of vitamin A at a given age, as indicated by the fact that they require a longer time (and make more growth) upon the vitamin A-free diet before the bodily store of this vitamin is sufficiently depleted (or "diluted" by the growth of the animal) for gain in weight to cease. It has also been found that when young from Diets A and B are depleted of their surplus vitamin A in the same manner, those from Diet B still show higher vigor and greater resistance to common infections throughout the subsequent experimental period. Thus among the animals used in one series of experiments it was found that 75 per cent of those from Diet A, and only 25 per cent of those from Diet B, showed visible signs of infection on autopsy at the end of the experimental period. We therefore consider it more satisfactory to use young from families on the diet containing the higher proportion of milk even though these require a slightly longer depletion period.

In chemical terms the higher proportion of milk means, among other things, a diet richer in calcium, in certain of the amino acids, and in vitamin G (the more thermostable component of the vitamin B complex), as well as in vitamin A. It is hoped that the separate significances of these several factors may be shown in subsequent papers. Meantime it may be said that the experience of this laboratory seems to indicate that the vitamin A content of the family diet influences the vitality of the young, not only in respect to their bodily stores of this substance, but in some other way or ways as well.

Basal Diet.

The basal vitamin A-free diet, to which the young rats to be used for these experiments are transferred when 28 or 29 days old, consists of: purified casein, 18 per cent; dried yeast, 10 per cent; Osborne-Mendel[2] salt mixture, 4 per cent; sodium chloride, 1 per cent; corn-starch, 67 per cent (diet, Laboratory No. 379 a).

Sources of Vitamin D.

The antirachitic or vitamin D requirement was met (1) by irradiating the animals with a mercury vapor quartz lamp, (2) by irradiating the corn-starch entering into the basal diet, or (3) by adding to the basal diet 0.05 per cent of irradiated commercial cholesterol; as well as by the bodily store of vitamin D which the animals contained when separated from the mother and the family dietary at 28 days of age, and which *under our conditions* does not become depleted until long after the depletion of vitamin A if the young animals are kept upon a diet supplying neither vitamin A nor D.

Of the three methods just mentioned for supplementing the bodily store and insuring an abundant supply of vitamin D throughout the experiment for the determination of vitamin A, our experience inclines us to favor the feeding of irradiated commercial cholesterol or other form of irradiated ergosterol with, or as a supplement to, the basal diet; but it cannot be said that either of the other methods used was in any way consistently detrimental in our experience.

[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

Depletion and Test Periods.

Young rats from our colony, selected and fed as above described, usually gain about 35 to 50 gm. in body weight before growth ceases upon the basal diet; *i.e.*, during the depletion period. When growth has definitely ceased under conditions which extended experience indicates to mean the depletion of the bodily store of vitamin A, the animal usually also showing incipient signs of vitamin A deficiency, the depletion period is considered complete; each animal is placed in a separate all metal cage with raised screen floor to prevent access to excreta, and the test period or experimental period proper is begun.

Usually, of each litter of animals used in work of this kind, one or more is continued on the basal diet without supplement as a negative control which is observed daily for symptoms of the characteristic pathology of vitamin A deficiency. The experience thus gained ultimately enables the worker to detect these symptoms in their incipiency and thus to begin the test period a few days after growth has ceased on the basal diet and about 1 day before the appearance of distinctly sore eyes, the object being to have the animals thoroughly depleted of surplus vitamin A but not in a seriously pathological condition when the experimental or test period is begun. From this point onward, different test animals are fed different amounts of the food under investigation to determine what daily allowance of the food will furnish that amount of vitamin A just sufficient to permit the rat to grow at an average rate of 3 gm. per week; while the behavior of the negative controls gives assurance of the representative character of the animals which are being employed.

The nutritional requirement of the standard test animal thus becomes the basis for the measurement of the vitamin values of the foods or other materials tested; and, in the interest of accuracy of such determinations of vitamin A, we have studied the records of large numbers of our experiments to ascertain the extent to which the gains in weight during the test period are influenced by the sex of the experimental animal, or its size at the beginning of the test period.

Relation of Size of Experimental Animal.

The relation of the size of the experimental animal at the beginning of the test period to its gain in weight during that period

has been investigated by means of a critical study of the data of experiments in which two or three animals of the same sex but different size had been fed the same amount of vitamin A; and, in the cases here considered, always such an amount as to induce a gain at somewhere near the "unit" rate of 3 gm. per week. The data were grouped according to whether the animal at the end of the depletion and beginning of the test period was small (under 75 gm.), medium-sized (75 to 100 gm.), or large (over 100 gm.). In 81 cases it was possible to compare the results obtained upon a medium-sized animal with those of a small animal of the same sex; in twenty-eight cases medium-sized and large animals could be similarly compared; and in thirteen cases we had sets of three strictly comparable animals, one small, one medium-sized, and one large. Thus grouped, the data when averaged yielded the comparisons shown in Table I.

TABLE I.

Comparison of Gains of Small, Medium-Sized, and Large Test Animals When Fed the Same Amounts of Vitamin A (about Sufficient to Support a Unit Rate of Gain during Test Period).

Size of rat.	No. of cases.	Average weight at beginning of test period.	Average gain in 8 wks. test period.	Differences and their probable errors.
		gm.	*gm.*	*gm.*
Small.	81	65	26 ± 0.6	6 ± 0.9
Medium.	81	87	20 ± 0.7	
Medium.	28	89	25 ± 1.1	
Large.	28	115	15 ± 1.9	10 ± 2.2
Small.	13	69	30 ± 1.9	8 ± 2.5
Medium.	13	89	22 ± 1.7	
Large.	13	113	14 ± 1.6	8 ± 2.3

The figures in the last column of Table I show the extent to which, in each of the three groupings, the average gain (for 8 weeks) of the large or small animals differed from that of the directly comparable animals of medium size, together with the probable error of this difference. The differences are from 3 to 7 times as large as their probable errors, and are undoubtedly significant.

Hence it appears that in order to obtain results of the highest accuracy in quantitative determinations of vitamin A by the rat

growth method, only such animals as do not differ greatly among themselves in size at the end of the depletion period should be used. For conditions such as those of our laboratory, a minimum limit of 70 or 75 gm. and a maximum limit of 100 gm. at the end of the depletion period would seem to be desirable.

There is clearly a tendency toward an inverse relationship between the body weight of the animal and its rate of gain upon a given limited allowance of vitamin A. Undoubtedly, too, this may also be stated conversely; *i.e.*, that among animals of the same species and age the amount of vitamin A required to support a given rate of gain tends to vary with the size of the individual. This corresponds with the similar relationships previously established in the case of vitamin B.[3,4]

Comparison of Males and Females as Experimental Animals.

The earlier experience of this laboratory,[1] which indicated that males and females may be used interchangeably in determinations of vitamin A by the method here employed, with gains of about 3 gm. per week in the test period, has now been confirmed by the data of large numbers of experiments made in such manner as to permit of direct comparison of the quantitative results yielded by the two sexes.

Thus the average gain shown during the 8 weeks test period by 148 males was 24.6 gm., and by 148 directly comparable females was 22.3 gm.

Statistically, the average difference of 2.3 gm. (or slightly less than 0.3 gm. per week) is probably, though not certainly, significant. Practically, it seems so small as to be safely negligible, although for investigations in which an extraordinary degree of refinement is sought it might be slightly preferable to work entirely with animals of the same sex.

Probably any possible slight advantage theoretically attributable to discrimination between the sexes will actually, in experiments of this kind, be outweighed by the advantages of being able to make greater use of litter mate controls when both sexes are employed.

[3] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1922, liv, 739.

[4] Sherman, H. C., and MacArthur, E. H., *J. Biol. Chem.*, 1927, lxxiv, 107.

The very slight tendency toward more rapid growth on the part of males than of females of the same age and nearly the same size, will in practise tend to be offset by the larger size which the males will usually have attained at the beginning of the test period, when the animals will usually be about 2 months old. This equalizing influence can readily be further availed of, at the cost of rejection of very few animals, by specifying a slightly larger standard size for male than for female rats at the end of the depletion and beginning of the test period. Any laboratory might, for example, decide that only females between 70 and 95 gm. or males between 75 and 100 gm. should be used interchangeably as standard test animals for quantitative determinations of vitamin A by the rat growth method.

It should, however, be carefully noted that all the foregoing discussion of the relation of sex to rate of gain, refers to experiments in which the level of feeding of the vitamin A-containing food or other material to be tested is such as to induce about unit growth, *i.e.* gain in weight of about 3 gm. per week during the test period. Much larger differences are to be expected if the level of vitamin A feeding is considerably higher, for in this case something approaching a normal rate of growth may become possible, and, as is well known, the normal growth curve of the male rat is distinctly steeper than that of the female at this age. Thus when we fed about twice the amount of vitamin A-containing food required for unit growth, the average gain during the 8 weeks test period was: for 117 males, 57 gm.; for 117 females, receiving the same amount of vitamin A, 42 gm.

Length of Experimental Period.

The practise of this laboratory, and, we judge, of a large proportion of research workers elsewhere, has been to continue the test period for 8 weeks. Laboratories devoted to control work upon cod liver oil commonly use a test period of 5 weeks and this was, therefore, incorporated in the United States Pharmacopoeia method of determining vitamin A values. With both practises in use, and resulting data recorded in terms of the same unit, it becomes important to know whether this difference in length of test period will influence either the uniformity of the results obtained or their average numerical values.

Averaging the results of 104 of our experiments in which the test animals showed about unit growth, we find at the end of the usual 8 weeks test period a gain of 25.89 gm. (or 3.24 gm. per week) with a coefficient of variation of 26 per cent. These same experiments averaged at the end of the 5th week of the test period show a gain of 18.12 gm. (or 3.62 gm. per week) with a coefficient of variation of 45 per cent. Thus the shortening of the test period to 5 weeks would have resulted in findings somewhat more variable and tending to be higher. The average difference of 0.38 gm. per week in rate of gain is about one-sixth of that which results from doubling the vitamin intake under these experimental conditions; so that the shortening of the test period from 8 weeks to 5 weeks might be expected, as an average tendency, to increase by about one-sixth the numerical ratings of vitamin A values of materials thus tested.

Desirable Rate of Growth during Test Period.

The English investigators to whom we are indebted for the fundamentals of this method of determining vitamin A recommended that the least practicable gain in weight during the test period be taken as the basis for quantitative comparison.[5] After somewhat extended experience in this laboratory we adopted a rate of gain of 3 gm. per week during the test period as a satisfactory standard or unit rate; and this same unit rate has also been adopted by the United States Pharmacopoeia. A lower level of vitamin A intake tends to result in greater mortality or more frequent cases of serious illness among the experimental animals; and at higher levels of intake the rates of gain are not increased in full arithmetical proportion, as was plainly shown by data of Sherman and Munsell,[1] so that quantitative comparisons at higher rates of gain necessarily make the method less delicate.

Others have more recently suggested[6] that the adoption of a higher level of vitamin intake and rate of gain during the test period might make the method more satisfactory by inducing a more vigorous condition in the test animals. While undoubtedly the higher level of vitamin A intake does result in more

[5] Drummond, J. C., and Coward, K. H., *Biochem. J.*, 1920, xiv, 661.

[6] Dutcher, R. A., Honeywell, H. E., and Dahle, C. D., *J. Biol. Chem.*, 1927, lxxv, 85.

vigorous animals, our experience has not, on the whole, confirmed the view that the method is thereby made more accurately quantitative. As already noted, there is unavoidably a loss in delicacy of response to small increments of vitamin intake as the level of vitamin feeding is raised. We also find, as noted above, that the higher rates of growth, induced by higher levels of experimental feeding of the vitamin-containing material under investigation, are attended with less regular results as between male and female test animals. For young rats from our colony subjected to the régime already outlined in this paper, a level of vitamin A feeding which induces a gain of 3 gm. per week during the test period seems adequate for the maintenance of a sufficient degree of health for the purpose of these experiments; and it permits of more delicate testing than does comparison at a higher level because the higher the level of vitamin intake the less is the response induced by a given small increment in this intake. It was still conceivable that the higher level of vitamin feeding and consequent rate of growth might, through inducing a higher degree of health, result in more uniform findings if the experiments are so controlled as to prevent the introduction of variations due to the differing responses of the sexes at the higher levels. When this was done in 104 cases directly comparable with the like number of cases mentioned in the preceding section of this paper, our experiments showed coefficients of variability of 32 and 30 per cent at the ends of the 5th and 8th weeks, respectively, when the level of vitamin intake during the test period was twice that of the experiments above mentioned. Here the variability is found to be slightly (perhaps not significantly) greater at the higher level when the tests are continued for 8 weeks, while at this higher rate of growth there was less increase of variability when the test period was shortened to 5 weeks. On the whole, we find no certain gain in uniformity to balance the undoubted loss in delicacy when the higher rate of growth is employed as a standard by means of which to make quantitative comparisons of vitamin A values.

SUMMARY.

In experiments upon the quantitative determination of vitamin A by the rat growth method, the diet fed to the mother, and available also to the young until separated from her at the age of 4

weeks, was found to influence markedly the vitality of the young as shown by susceptibility to infection during the test period, even though variations in store of vitamin A in the body were eliminated by means of a preliminary depletion period.

Other conditions being the same, the larger the experimental animal at the beginning of the test period the less is its rate of gain upon a given limited intake of vitamin A. Doubtless this may with equal accuracy be stated in the converse form, that is, among animals of the same species and age, the amount of vitamin A required to support a given rate of gain in weight tends to vary with the size of the individual.

Under the conditions described, with a rate of gain of about 3 gm. per week during the test period, male and female rats may be used interchangeably as test animals.

In experiments in which the rate of gain was approximately the standard unit rate of 3 gm. per week, it was found that to have shortened the test period from 8 weeks to 5 weeks would have resulted in slightly more variable findings and a higher average rate of gain per week. The shorter test period therefore tends to give the material tested a higher numerical rating in vitamin A value.

The suggestion that a considerably higher rate of gain during the test period, such as about 6 gm. per week, be adopted as the basis for quantitative work in the determination of vitamin A by the rat growth method, is studied by examination of the data of over 100 experiments at each of the two levels in question. While attractive in some respects, this suggestion, of a change to a standard rate of gain distinctly higher than the present unit rate of 3 gm. per week during the test period, is not, on the whole, found desirable, because it decreases the delicacy of the method without apparently increasing the accuracy. The present investigation therefore supports the continued use of the present unit rate of 3 gm. per week of gain in body weight in a standard test animal depleted of surplus bodily store of vitamin A, as the basis for quantitative work in the determination of vitamin A by the rat growth method.

THE COPPER CONTENT OF MILK.

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The presence of copper in cow's milk has been reported by several investigators (1–5). The most extensive investigation was that of Supplee and Bellis (2) who determined the copper content of freshly drawn milk of a large number of cows fed on different rations. They found that the copper content per liter varied from 0.2 to 0.8 mg., with no apparent correlation between copper content and ration. The variation in results obtained by investigators in widely different localities suggests the possibility of the influence of the copper content of native feed products. The analyses in this laboratory of milk collected from four states would indicate, however, that we can expect quite constant copper content of raw cow's milk. The copper content of milk of the sheep and of the goat was found to be about the same as that for the cow. The copper content of goat milk was low in all cases but always easily detectable notwithstanding the report to the contrary by Titze and Wedemann (6).

EXPERIMENTAL.

Raw Cow's Milk.—The samples of milk were collected in glass containers, directly from the cows. The possibility of contamination by copper during the analysis was always checked by means of equivalent quantities of copper-free water which were treated in the same manner as the milk. The copper was determined by the xanthate colorimetric method as modified by Supplee and Bellis (2). Each determination in Table I represents the average of three determinations of 100 cc. each of the milk from a given cow. Apparently the possible variation in copper content of native feeds, such as the grass in Kentucky (7), has little or no effect.

Raw Sheep Milk.—Samples of milk from a local herd gave the results recorded in Table II. The copper content is in very good agreement with that of cow's milk.

TABLE I.

Copper Content of Raw Whole Cow's Milk.

Herd by locality.	Animal No.	Copper.
		mg. per l.
Iowa.		
Mt. Vernon.	1	0.43
	2	0.42
	3	0.38
Cedar Rapids.	1	0.42
	2	0.45
	3	0.49
Luzerne.	1	0.49
	2	0.52
South Dakota.		
Manfield.	1	0.43
	2	0.42
Montana.		
Willard.	1	0.28
	2	0.26
Kentucky.		
Catlettsburg.	1	0.37
	2	0.40

TABLE II.

Copper Content of Raw Whole Sheep Milk.

Animal No.	Copper.
	mg. per l.
1	0.45
2	0.50
3	0.50

Raw Goat Milk.—Samples were collected from a local herd of Toggenburg goats. The copper content, as indicated in Table III, is much lower than that of cow's milk.

Processed Cow's Milk.—The copper content of milk that has been passed through a manufacturing process may vary between wide limits as shown in Table IV. The samples were analyzed

TABLE III.

Copper Content of Raw Whole Goat Milk.

Animal No.	Copper.
	mg. per l.
1	0.22
2	0.20
3	0.19
4	0.25
5	0.20
6	0.19

TABLE IV.

Copper Content of Processed Milk.

Product.	Factory No.	Sample No.	Copper.
			mg. per l.
Pasteurized whole milk.	I	1	0.60
		2	0.70
		3	0.63
	II	1	1.60
		2	1.50
		3	1.60
Buttermilk.	II	1	2.50
		2	2.45
		3	2.40
Concentrated milk.	III	1	2.32
	IV	1	1.80
	V	1	1.80
	VI	1	2.70

as described above. The concentrated milk samples were four common brands of condensed or evaporated milk. In all cases the copper was greater than could likely be accounted for in the

original raw product. The variation in copper content can be traced to the amount of copper surface exposed to the milk product.

BIBLIOGRAPHY.

1. Fluerent, E., and Levi, L., *Bull. Soc. chim.*, 1920, xxvii, 441.
2. Supplee, G. C., and Bellis, B., *J. Dairy Sc.*, 1922, v, 455.
3. Rice, F. E., and Miscall, J., *J. Dairy Sc.*, 1923, vi, 26.
4. Hess, A. F., Supplee, G. C., and Bellis, B., *J. Biol. Chem.*, 1923, lvii, 725.
5. Bertrand, G., *Bull. soc. hyg.-aliment.*, 1920, viii, 49.
6. Titze, C., and Wedemann, W., *Arb. k. Gsndhtsamte.*, 1911, xxxviii, 125.
7. McHargue, J. S., *Ind. and Eng. Chem.*, 1927, xix, 274.

ON THE QUESTION OF THE SPECIFICITY OF THE INTRACELLULAR DEHYDROGENASES.

I. THE DEHYDROGENASE OF CUNNER MUSCLE.

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INTRODUCTION.

When food substances are burned in the tissues, the process may be one of direct addition of active oxygen to the food molecule, or of oxidation through the withdrawal of hydrogen from the food substance and its subsequent union with oxygen. The latter method was first discussed by Wieland (7) and later demonstrated by Thunberg and his school in a long series of papers which have been reviewed briefly by Hopkins (3) and in detail by Ahlgren (1). All tissues thus far tested contain a dehydrogenase (or dehydrogenases) capable of transferring hydrogen from certain donators to methylene blue, oxygen, or other receptors. The most complete observations have been made upon frog tissues, but considerable work has been done upon the tissues of the guinea pig and the muscle of man, rabbit, horse, dove, snake, goldfish, lobster, certain seed plants, and *Bacillus coli.*

The question at once arises as to whether all the donators are attacked by a single dehydrogenase, or whether each one has its own specific enzyme. Thunberg and his school have certain evidence pointing toward the latter possibility. Thus, the crystalline lens of the eye (guinea pig) attacks several donators but does not affect succinic acid, a donator readily attacked by other tissues (2). In the aging of sterile human muscle, power to attack lactic acid falls rapidly on the 1st day and then holds firm, power to attack citric acid is gradually diminished from the 1st day onward, while power to attack succinic acid is little affected in 5 days (5). After exposure to extremes of temperature (−70° and +60°)

power to attack succinic acid is less affected than power to attack citric acid (6). Again grinding the tissue destroys its power to oxidize citric acid but does not greatly diminish its power to oxidize succinic acid (1). Extracts thus far made are rich in succinic dehydrogenase, but attack other donators less readily than the tissue itself. Perhaps the best evidence for Thunberg's theory of specificity is found in the fact that when frog muscle is added to a mixture containing optimal amounts of two donators (succinic and lactic, or succinic and glycerophosphoric acids), the reaction is more rapid than if only one of the donators is present, a result difficult to interpret unless there are at least three dehydrogenases (1).

The opposite point of view is taken by Wishart (8) who tested the decolorization time of a muscle extract upon optimal amounts of donators used singly and in pairs. Wishart observed no indication of an additive effect, but the value of his observations is lessened by the fact that he worked with a rather uncertain extract instead of with the muscle itself. Quastel and Wooldridge (4) in a long series of experiments upon *Bacillus coli* also conclude that, at least in that organism, there is only one dehydrogenase and that one not specific. It is of course possible that the dehydrogenase system is not identical in all organisms.

Evidently the solution of the problem demands further evidence. It is therefore proposed to test the question in various ways on the muscles of two organisms, fish and frog. In this paper experiments upon the muscle of the cunner (*Tautogolabrus adspersus*) are reported, in which optimal concentrations of various donators were used singly and in pairs, and the time measured in which a given quantity of methylene blue was completely decolorized *in vacuo*. If there is only one dehydrogenase, the decolorization time with a pair of donators will be no shorter than with the more active of them singly. If there are several specific dehydrogenases, each attacking a special donator, the decolorization time must be shorter when the donators are used in pairs, for the two reactions will proceed independently and the effect will therefore be additive.

Method.

In general the standard method used by Thunberg was followed (1,6). The fish were stunned by a blow, decapitated, and skinned.

The heavy dorsal trunk muscles were removed, cleared of the bones. The muscle mass, weighing some 10 gm., was finely divided by cutting for 15 minutes with a curved scissors. It was then shaken twice, for 15 minutes each time, with 20 times its weight of redistilled water in order to remove the donators and excess salts. After each washing it was filtered on cheesecloth and perfused with 50 cc. of K_2HPO_4 0.075 M. The use of a buffer rinse is a departure from the usual method and is useful in maintaining the activity of the enzyme at a high level. The washed tissue was kept in a covered watch-glass in the refrigerator until just before use.

Stock solutions of the reagents (except methylene blue) were made daily with fresh, redistilled water. The content of each tube was as follows:

0.05	cc.	methylene blue 1:5000.
0.15	"	K_2HPO_4 0.5 M.
0.10	"	donator 0.1 M (neutralized with KOH).
(0.10	"	" 0.1 ") " " "
0.60	"	H_2O.
1.0	"	total fluid.
0.2	gm.	washed muscle.

Each tube was evacuated as soon as the muscle had been added. Evacuation requires 30 to 60 seconds with a water pump if the pressure is above 50 pounds per square inch. It is important to continue the evacuation until all bubbles of gas are removed from the tissues. The tube is continually tapped on a cork block to keep the tissues from sticking to the walls and to prevent foaming. When warming the tube in one's hand no longer leads to the appearance of bubbles, evacuation is complete.

The time was noted when the tube was placed in the bath and again when decolorization was complete. The temperature was kept at 36°. The bath is essentially like the one described by Ahlgren (1), but differs from his in having the stirrer suspended from above the center of the bath and in being heated by blackened carbon bulbs. The arrangement was worked out by the apparatus department of the Marine Biological Laboratory at Woods Hole.

TABLE I.

Decolorization Time, in Minutes, of Donators Alone and in Pairs.

Acid.					
Aug., 1926	17	29	30	19	15
Citric	45	23	43		
" and succinic	31	15	16		
Succinic	30	16	13		
" and glycerophosphoric	44	20	17		
Glycerophosphoric	80	40	34		
Citric and glycerophosphoric	67	27	32		
"	63	23	44	45	59
Lactic and citric	47			37	66
"	45			23	59
" and succinic	40			22	50
Succinic	40			22	39
Lactic and glycerophosphoric	56				60
Glycerophosphoric	56			28	60
Acetic	99			34	73
" and citric	73			55	70
Citric	60			42	70
Acetic and succinic				23	
Succinic	42			24	
Acetic and glycerophosphoric	78			40	
Glycerophosphoric	78	39	49	35	
" and *l*-malic		41	57		
l-Malic		38	58		
" and citric		31	48		
Citric		31	47		
l-Malic and succinic		18	15		
Succinic		19	13		

TABLE II.

Effect of Buffer Rinse upon Decolorization Time, in Minutes.

Acid.	6 hrs. after washing.	
	Rinsed.	Not rinsed.
Succinic	25	38
Glycerophosphoric	60	80
Citric	70	100

Results.

Table I shows typical results. It is evident at once that when two donators at optimal concentration are combined, the decolorization is not any quicker than when the more active of the two is used alone. There is consequently no evidence whatever of an additive effect. In the single experiment performed with acetic and butyric acids, the same result was observed. The effect of the buffer rinse in keeping the activity of the tissue at a high level is shown in Table II.

CONCLUSIONS.

In cunner muscle a single dehydrogenase is present, which is capable of oxidizing anaerobically several donators; *viz.*, succinic, citric, glycerophosphoric, lactic, *l*-malic, acetic, and butyric acids.

Rinsing the chopped muscle in the buffer after washing it in distilled water improves the activity of the enzyme.

BIBLIOGRAPHY.

1. Ahlgren, G., *Skand. Arch. Physiol.*, 1925, xlvii, suppl.
2. Ahlgren, G., *Skand. Arch. Physiol.*, 1923, xliv, 196.
3. Hopkins, F. G., *Skand. Arch. Physiol.*, 1926, xlix, 33.
4. Quastel, J. H., and Wooldridge, W. R., *Biochem. J.*, 1927, xxi, 148.
5. Rosling, E., *Skand. Arch. Physiol.*, 1924, xlv, 132.
6. Thunberg, T., *Skand. Arch. Physiol.*, 1920, xl, 1.
7. Wieland, H., *Ber. chem. Ges.*, 1912, xlv, 484, 2606.
8. Wishart, G. M., *Biochem. J.*, 1923, xvii, 103.

STUDIES IN THE METABOLISM OF THE BILE.

II. THE SEQUENCE OF CHANGES IN THE BLOOD AND BILE FOLLOWING THE INTRAVENOUS INJECTION OF BILE OR ITS CONSTITUENTS.*

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Knowledge of the physiologic mechanisms concerned in the formation and excretion of the bile is essential to the understanding of changes observed clinically in diseases of the liver or biliary tract. This is particularly true in the study of those conditions in which there is retention or accumulation of biliary constituents in the blood. In such conditions the institution of suitable treatment must be based on an understanding of the manner in which these materials are normally removed from the blood stream. We have sought, therefore, to provide an experimental basis for future clinical study by an investigation of the sequence of changes in the blood and bile following the intravenous injection, both of bile and of its two chief constituents, the bile acids and bilirubin.

The rapid disappearance of bile or bile salts from the blood stream after their intravenous injection was noted by Kühne (15) and Huppert (13). The methods at their disposal, however, did not permit detailed study of the process. The accurate study of changes in the bilirubin content of the blood has been made possible by the work of van den Bergh (2) in the development of the test that bears his name. The work of Aldrich (1) on a quantitative modification of the Pettenkofer reaction applicable to the analysis of small quantities of blood permits us to estimate the bile acids in a similar fashion.

* Reported in part before the American Physiological Society, Rochester, New York, April, 1927, and the Physiological Society, Edinburgh, August, 1927 (8, 9).

We have used these newer methods to study the elimination of bile or its constituents following their intravenous injection. Two series of experiments were carried out. The first was planned to compare the rate at which bile acids, bilirubin, and bile disappeared from the blood stream and to determine the effect of varying doses. The second was planned to compare the rate at which the bile acids and bilirubin were eliminated in the bile and the effect of each on the volume and composition of the latter.

Method.

The bile used for injection was obtained from hospital patients at operation. The absence of infection in such specimens was shown by the associated bacteriologic studies of Judd, Mentzer, and Parkhill (14). Purified samples of bilirubin were obtained from the Eastman Kodak Company. Commercial samples of bile salts, obtained from Armour and Company, which are mixtures of sodium glycocholate and taurocholate were used. These were analyzed as described by Aldrich and Bledsoe (1) and the dosage given was calculated on the basis of the content of bile acids.

Dogs were used in all experiments. In the study of the changes in the blood, the desired amount of bile, bilirubin, or bile salts, calculated in terms of mg. for each kilo of body weight, was dissolved in a physiologic solution of sodium chloride and injected intravenously at a uniform rate by means of a modified Woodyatt pump. A period of 1 hour was used for the injections which were made into the saphenous vein under local anesthesia. Specimens of blood for analysis were withdrawn from the jugular vein at the beginning of the experiment. Subsequent samples were taken during the period of injection and the course of recovery. The amounts given, while large in proportion to the usual daily output of a dog with a biliary fistula, were usually well tolerated. Some animals were restless and vomited during the injection, but recovery was complete by the following day. The largest doses, however, were toxic and caused death of the animal in from 12 to 48 hours.

The study of the composition of the bile was carried out on dogs under amytal narcosis. The gallbladder was removed and a cannula placed in the common bile duct. Samples of the hepatic

TABLE I.

Elimination of Bile Acids from Blood Following Timed Intravenous Injection.

Experiment No.	Date.	Dose.	Normal before injection.	Injection period, min.						Elimination period, min.							
				10	20	30	40	50	60	10	20	40	60	80	100	120	150
			Glycocholic acid, mg. per 100 cc.														
	1926	*mg. per kg.*															
1	Feb. 3	75	4.8	4.5	5.2	4.8	5.6	5.6	5.8		5.1		4.3		4.8	4.4	4.8
2	" 10	150	4.3	4.6	9.2	10.5	10.6	11.8	12.1	7.8	5.4	6.0	5.6	5.4	4.4	4.8	4.3
3	Mar. 25	250	7.6	17.6	23.0	23.0	28.7		43.5	19.1	10.0	10.2	8.7	8.2	8.8	7.8	8.9
4	" 12	300	6.8	29.4	47.6	65.8	65.6	74.0	80.0	60.6	40.8	20.2	12.5	9.0	8.8	8.1	7.6
5	Apr. 23	350	5.8	35.7	42.8	62.8	81.5	94.0	103.4	81.5	63.3	38.1	15.8	9.4	9.1	8.5	7.1
6	Mar. 19	400	6.8	47.2	76.3		95.2	130.0	147.0	Died.							

bile so obtained were collected at half hour intervals. Usually two preliminary samples were collected to establish the normal rate of flow of the bile. Commercial preparations of bile salts were then injected intravenously in a dosage equivalent to from 100 to 125 mg. of glycocholic acid for each kilo of body weight and the collection of bile continued for a period of 6 hours. Samples of blood were taken from the jugular vein at appropriate intervals and the bile acids determined by the method of Aldrich (1). The concentration of bilirubin and bile acids in the specimens of bile was determined by the methods previously described (10).[1] Kühne (15), Huppert (13), Hoppe-Seyler (11), Brakefield and Schmidt (4), and others have reported the appearance of bile acids in the urine following their intravenous injection. To prevent such loss through the urine, both kidneys were removed at the beginning of the experiment. Control experiments were made on fasting animals and the effects of the injection of hemoglobin and bilirubin were studied for contrast with the action of the bile salts.

To control the effect of the anesthetic and of the operative procedures a second group of experiments was performed on a dog with a permanent biliary fistula. In the preparation of this fistula the gallbladder was removed and the common bile duct intubated according to the technique reported by Rous and McMaster (16). Samples were collected for analysis according to the same schedule as in the anesthetized dogs.

Rate of Elimination of Bile and Its Constituents from Blood.

Bile Acids.—When the sodium salts of the bile acids are injected intravenously under the conditions of these experiments there is rapid increase in the intensity of the Pettenkofer reaction given by the blood. Varying amounts of material equivalent to doses of from 75 to 400 mg. for each kilo were given. In all cases the dosage was considerably in excess of the usual production

[1] Glycocholic acid was used as a standard in the determination of the bile acids and the results are reported in terms of mg. of glycocholic acid for each 100 cc. of blood or in per cent of the bile. Dog bile normally contains only taurocholic acid. We did not attempt to differentiate between the two. The figures obtained should be increased by 10 per cent if a comparison on the basis of taurocholic acid is to be made.

of bile acids in an animal, as measured by means of a biliary fistula. The smallest dose, 75 mg. for each kilo, caused little if any change in the blood. With larger doses (Table I) the bile acid content of the blood was markedly increased. The height of this increase varied with the dose given. As soon as the injection was stopped the excess was removed from the blood with great rapidity. In Experiment 5 the level in the blood was increased to 103.4 mg. for each 100 cc. but this was reduced to 8.5 mg. within 2 hours. When smaller doses were given the content of the blood was not so greatly increased and the normal level was regained in a shorter time.

TABLE II.

Elimination of Bile Acids from Blood Following Rapid Intravenous Injection.

Experiment No.	Date.	Dose.	Normal before injection.	Time after injection, min.									
				5	10	15	30	45	60	90	120	150	180
			Glycocholic acid, mg. per 100 cc.										
	1926	*mg. per kg.*											
7	May 20	40	5.3	14.6	6.9	6.4	7.8	6.8	6.8	6.3	6.0	5.0	4.5
8	" 17	80	5.4	38.4	25.1	11.0	5.9	5.8	5.9	5.0	5.3	4.8	5.2
9	" 21	120	5.2	37.5	28.1	20.4	7.4	4.8	5.4		4.8	5.2	6.2
10	" 13	160	5.0	72.5	64.0	61.6	32.4	14.5	8.2	5.8	5.2	5.1	5.1
11	" 31	200	7.3	87.0	75.4	63.7	42.8	10.1	9.5	7.1	8.4	8.4	7.1
12	June 1	240	7.3	146.0	126.0	120.0	102.0	70.0	42.0	13.3	9.0	8.0	7.8

For purposes of comparison a similar group of experiments was made in which the bile acids were given at a more rapid rate. A 5 per cent solution was used and amounts of from 40 to 240 mg. for each kilo were injected within from 2 to 3 minutes. The changes in the bile acid content of the blood in these experiments are shown in Table II. As with the first group of experiments the increase of bile acids in the blood was dependent on the dose given. With equivalent doses, the shorter period of injection produced a much greater increase in the level in the blood. In Experiment 12 a level of 146 mg. for each 100 cc. of blood was reached after the injection of 240 mg. for each kilo of body weight. The disappearance of the excess of bile acids from the blood was

TABLE III.

Elimination of Bilirubin from Blood Following Timed Intravenous Injection

Experiment No.	Date.	Dose.	Normal before injection.	Injection period, min.				Elimination period, min.									
				15	30	45	60	15	30	45	60	90	120	150	180	210	240
			Bilirubin, mg. per 100 cc. serum.														
	1927	*mg. per kg.*															
13	Jan. 17	10	0.0	0.6	1.2	1.7	2.4	1.2	0.6	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
14	" 20	25	0.0	2.3	4.1	4.1	5.3	3.6	2.9	2.2	1.6		0.9	0.8	0.8	0.7	0.3
15	Mar. 9	35	0.0	6.1	8.8	11.2	17.4	17.0	16.0	15.5	14.2	12.8	12.7	11.9	10.1		
16	Feb. 14	50	0.0	10.4	15.3	20.0	23.1	22.1	18.5	17.2	16.5	11.7	9.7	7.4	5.7	4.8	4.3

quite as rapid as in the first experiments with slower injections and the concentration was reduced to 8 mg. 2½ hours later. The smaller doses produced less marked increases in the concentration and the excess was removed in a correspondingly shorter time.

In the experiments dealing with the excretion of bile acids in the bile, a standard dose of bile acids equivalent to 125 mg. of glycocholic acid for each kilo of body weight was rapidly injected. This produced an approximately tenfold increase in the blood over the normal Pettenkofer reading of from 5 to 6 mg. for each 100 cc. The disappearance of the excess was rapid, and, with the dosage used, the level in the blood decreased to one-half or one-third of the maximum in 30 minutes. The normal value was regained approximately by the end of the hour. In one experiment, samples of blood were taken from the portal vein. There was no significant difference between the amount of bile acids in this blood and in samples from the systemic circulation. We were unable to demonstrate bilirubinemia by means of the van den Bergh reaction in any of these experiments.

Bilirubin.—Bilirubin accumulated in the blood during its intravenous injection in much the same manner as the bile acids. Amounts varying from 10 to 50 mg. for each kilo of body weight were given. The effect varied with the dose. As shown in Table III, the injection of 10 mg. for each kilo of body weight resulted in a concentration of 2.4 mg. for each 100 cc. of serum, while a level of 23.1 mg. was attained after the administration of 50 mg. for each kilo of body weight. The excess of bile pigment readily left the blood stream although at a much slower rate than the bile acids. Considerable amounts of bilirubin were present in the serum 4 hours after the injection in all the experiments, except the one in which the smallest dose, 10 mg., was used. The curves of bilirubin elimination showed more variation than was found in the experiments with the bile acids. These variations were seemingly related to the general condition of the animal.

The injection of bilirubin alone was without effect on the intensity of the Pettenkofer reaction of the blood. The readings for the bile acids were unchanged during these experiments.

Mixtures of Bile Acid and Bilirubin.—One experiment (Table IV, Experiment 17) was made in which a mixture of bilirubin and bile salts was injected. In this experiment the increase in the

TABLE IV.

Elimination of Bile Acids and Bilirubin from Blood Following Timed Intravenous Injection of a Mixture or of Whole Bile.

Experiment No.	Date.	Material injected.	Content of material injected.	Dose.	Normal before injection.	Injection period, min.				Elimination period, min.									
						15	30	45	60	15	30	45	60	90	120	150	180	210	240
					Mg. per 100 cc. of blood or serum.														
	1927			*mg. per kg.*															
17	Apr. 16	Mixture of pure material.	Bile acids.	200	4.8	7.1	9.1	10.2	15.4	7.0	5.8	5.3	5.2	5.3	5.1	4.9		5.0	4.9
			Bilirubin.	25	0.0	3.0	3.6	4.7	5.9	4.0	3.9	3.2	2.3	2.1	1.6	1.3	0.5	0.7	0.3
18	Jan. 12	Whole bile.	Bile acids.	110	5.3	10.1	10.5	11.0	11.5	7.0	6.9	6.1		6.0	5.2	5.1	5.1		4.7
			Bilirubin.	5	0.0	0.8	1.3	1.5	1.9	1.0	0.8	0.7	0.7	0.3	0.0	0.0	0.0	0.0	0.0
19	Feb. 1	" "	Bile acids.	155	6.5	22.0	35.3	41.1	47.1	36.6	34.3	29.6	25.8	18.2	12.8	8.2	7.7	7.4	5.9
			Bilirubin.	15	0.0	2.2	3.8	5.1	5.8	5.1	4.9	4.8	4.2	3.8	3.0	2.7	1.7	0.6	0.0
20	Jan. 15	" "	Bile acids.	175	7.5	26.3	38.7	49.4	57.5	48.0	46.5	40.0	40.8	32.1	20.0	19.2	18.0	16.9	16.7
			Bilirubin.	15	0.0	3.9	5.7	7.9	9.1	7.5	7.3	7.0	6.9	6.7	5.4	5.4	5.3	4.9	4.8
21	" 18	" "	Bile acids.	200	7.0	36.0	63.0	63.0	70.0		62.4	55.0	54.6	53.2	50.0	48.0	45.6	45.3	42.5
			Bilirubin.	10	0.0	2.3	3.3	4.3	5.2	4.2	4.1	4.1	3.6	4.2	4.1	3.9		3.3	
22	Feb. 18	" "	Bile acids.	200	9.0	64.5	92.8	102.0	103.0	98.0	85.1	78.1	71.5	54.0	39.0	29.2	20.0	18.7	
			Bilirubin.	25	0.0	6.5	11.1	12.0	13.2	12.5	11.4	10.2	9.3	8.8	8.6	7.8	6.5	5.3	

concentration in the blood and the curve of excretion of each constituent of the mixture was essentially the same as when each was given alone.

Whole Bile.—Whole bile was diluted with a physiologic solution of sodium chloride and injected in the same manner as the solutions of bile salts or bilirubin. The changes observed, as recorded in Table IV, differ considerably from those that followed the injection of purified materials.

Bile containing an amount of bile acids equivalent to 200 mg. for each kilo of body weight increased the amount in 100 cc. of blood to 103 mg. in one instance (Experiment 22) and to 70 mg. in another (Experiment 21). Furthermore elimination was greatly delayed and the levels were 18.7 and 45.3 mg., respectively, at the end of 3½ hours. The same dose of bile salts in pure solution raised the reading in the blood to but 26.1 mg. and normal levels were regained in less than 2 hours. Similar results, although less striking, were obtained by the injection of bile containing smaller amounts of bile acids.

Analogous changes were observed in the behavior of the bile pigment. In Experiment 22, bile containing 25 mg. of bilirubin for each kilo of body weight was injected. This raised the level in the blood to 13.2 mg., and 5.3 mg. were still present at the end of 4 hours. The same dose of bilirubin when given alone (Table III, Experiment 14) provided a maximal concentration in the serum of 5.3 mg., and only 0.3 mg. was present at the end of the experiment. Similar results were obtained in the other experiments in which bile was injected.

Composition of Bile after Intravenous Injection of Certain of Its Constituents.

Bile salts given intravenously have a marked cholagogue effect. The increased rate of bile flow could be noted within 15 minutes although the maximal effect was present during the second collecting period (Tables V and VI). This maximal increase varied from 4 to 10 times the normal, the most marked change being observed in Experiment 23 (Table V) in which 15 cc. (1.1 cc. of bile for each kilo of body weight) were obtained during one 30 minute collection period. The period of maximal choleresis was of short duration and did not last more than 2 hours, although the bile flow

was usually somewhat augmented over the normal during the entire course of the experiment. On the other hand, in control experiments on fasting animals in which salt solution only was injected (Table VII), the bile flow decreased slightly during the

TABLE V.

Changes in Blood and Bile Following Intravenous Injection of Bile Acids.

Experiment 23. Weight of dog 13.3 kilos.

Time of collection.		Blood.		Bile.					Remarks.
		Bile acids.			Bilirubin.		Bile acids.		
Before injection.	After injection.	Jugular.	Portal.	Volume.		Total.		Total.	
hr.	*min.*	*mg. per cent*	*mg. per cent*	*cc.*	*mg. per cent*	*mg.*	*per cent*	*mg.*	
1		6.9		1.1	80	0.88	4.20	47	Control period.
½		6.1	5.7	1.9	72	1.37	3.05	58	
	5	64.0							52 cc. bile salt solution containing 1540 mg. bile acids injected intravenously. Total bile acids recovered 1885 mg., total bilirubin 28.59 mg.
	10	50.8	52.0						
	15	38.2							
	30	21.4	24.8	11.0	16	1.76	4.33	480	
	45	14.2							
	hrs.								
	1	8.9	7.5	15.0	13	1.95	4.84	730	
	1½	7.7		8.5	43	3.65	2.62	222	
	2	6.7	7.7	4.4	88	3.87	1.90	84	
	2½			3.5	96	3.36	1.90	67	
	3	5.5		3.4	100	3.40	1.90	65	
	3½			2.5	108	2.70	2.25	56	
	4	5.6	7.6	2.2	110	2.42	2.40	53	
	4½			2.0	111	2.22	2.97	59	
	5	6.2		1.3	144	1.82	2.97	39	
	5½			0.5	144	0.72	2.97	15	
	6	4.8	5.7	0.5	144	0.72	2.97	15	

first two periods but was relatively constant thereafter. This decrease, first observed by Schiff (17), is associated with the external drainage of the bile and consequent interruption of the normal enterohepatic circulation of the bile acids.

The concentration of bile acids in the bile increased but to a much less degree than the increase in volume of the bile secreted. The concentration in the normal hepatic bile obtained during the preliminary periods varied from 1.80 to 6.04 per cent. After the

TABLE VI.

Changes in Blood and Bile Following Intravenous Injection of Bile Acids.

Experiment 24. Weight of dog 12.2 kilos.

Time of collection.		Blood.	Bile.					Remarks.
				Bilirubin.		Bile acids.		
Before injection.	After injection.	Bile acids.	Volume.		Total.		Total.	
hr.	*min.*	*mg. per cent*	*cc.*	*mg. per cent*	*mg.*	*per cent*	*mg.*	
1			1.0	117	1.17	4.9	49	Control period.
½		6.3	0.6	117	0.70			
	5	56.1						46 cc. bile salt solution containing 1400 mg. bile acids injected intravenously. Total bile acids recovered 1925 mg., total bilirubin 39.79 mg.
	10	50.6						
	15	38.9						
	30	22.4	9.3	23	2.14	5.95	554	
	45	11.5						
	hrs.							
	1	10.9	12.6	16	2.02	4.65	586	
	1½	6.8	5.4	46	2.48	4.10	221	
	2	6.1	4.4	77	3.38	3.53	155	
	2½		2.6	113	2.94	3.54	92	
	3	5.8	2.3	137	3.15	2.02	46	
	3½		2.1	147	3.09	1.57	33	
	4	5.7	4.3	134	5.75	1.18	51	
	4½		4.6	132	6.06	1.11	51	
	5	5.5	4.3	68	2.92	1.02	44	
	5½		4.6	62	2.85	1.00	46	
	6	5.4	4.6	64	2.94	1.00	46	

injection of bile salts the maximal values varied from 4.33 to 6.00 per cent. This maximal value was attained during the 1st hour; later the concentration of bile acids diminished and minimal values of 1.00 to 2.94 per cent were reached during the latter

portion of the experiment. Similar changes in concentration were observed during the control experiments.

The effect of the intravenous injection of the bile acids on their excretion in the bile is best shown when the total quantity rather than the concentration is studied. During the preliminary

TABLE VII.

Changes in Blood and Bile in Control Experiment.

Experiment 25. Weight of dog 11.1 kilos.

Time of collection.		Blood.	Bile.					Remarks.
Before injection.	After injection.	Bile acids.	Volume.	Bilirubin.		Bile acids.		
					Total.		Total.	
hr.	*min.*	*mg. per cent*	*cc.*	*mg. per cent*	*mg.*	*per cent*	*mg.*	
1		1.0	0.7	173	1.21	4.45	31	Control period.
½		5.0	0.3	183	0.55	4.50	13	
	5	4.4						35 cc. physiologic solution of sodium chloride injected intravenously. Total bile acids 209 mg., total bilirubin 15.80 mg.
	10	4.5						
	15	4.2						
	30	5.0	0.2	311	0.61	5.00	10	
	45	5.0						
	hrs.							
	1	5.2	0.4	315	1.26	6.40	25	
	1½	5.3	0.4	294	1.18	5.08	20	
	2	4.3	0.4	375	1.50	4.10	16	
	2½		0.4	379	1.50	4.80	19	
	3	4.5	0.3	400	1.20	5.40	16	
	3½		0.4	365	1.45	5.10	20	
	4	4.4	0.5	433	2.16	5.17	26	
	4½		0.4	390	1.56	4.45	18	
	5	4.2	0.5	320	1.60	2.78	14	
	5½		0.5	276	1.38	2.82	14	
	6		0.4	98	0.40	2.82	11	

periods of the various experiments, the amount varied from 13 to 60 mg. (1.2 to 4.4 mg. for each kilo of body weight) for each 30 minute period depending on the condition of the animal. In the control experiments the output of bile acids was greatest during the first two periods (Table VII, Experiment 25). There-

TABLE VIII.

Changes in Blood and Bile of Animal with Permanent Biliary Fistula.

Time of collection. Before injection.	Time of collection. After injection.	Blood. Bile acids.	Bile. Volume.	Bilirubin.	Bilirubin. Total.	Bile acids.	Bile acids. Total.	Remarks.
hr.	*min.*	*mg. per cent*	*cc.*	*mg. per cent*	*mg.*	*per cent*	*mg.*	
Experiment 28. Weight of dog 14.6 kilos. Changes following the intravenous injection of bile acids.								
1		6.1	7.4	16	1.17	2.34	173	Control period.
½		6.0	6.5	21	1.42	1.28	83	
	5	37.2						46 cc. bile salt solution containing 1460 mg. bile acids injected intravenously. Total bile acids recovered 1304 mg.
	10	32.0						
	15	21.3						
	30	10.1	15.1	14	2.08	3.60	540	
	45	7.3						
	hrs.							
	1	6.6	8.5	20	1.66	2.42	206	
	1½	6.5	7.3	38	2.75	1.55	113	
	2	6.5	6.3	48	3.00	1.02	64	
	2½		6.2	71	4.43	0.93	58	
	3	6.1	6.1	54	3.30	0.88	54	
	3½		4.8	60	2.90	0.87	42	
	4	6.3	5.1	62	3.20	0.87	44	
	4½		5.3	56	3.00	0.91	48	
	5	5.9	5.0	61	3.05	0.92	46	
	5½		5.0	61	3.00	0.92	46	
	6	5.7	4.6	56	2.60	0.94	43	
Experiment 29. Weight of dog 14.6 kilos. Control experiment.								
1			13.0	40	5.20	1.63	181	Control period.
½		6.7	8.0	44	3.50	1.39	111	
	½		8.2	67	5.55	1.00	82	29 cc. salt solution injected intravenously. Total bile acids 627 mg.
	1	6.5	7.8	91	7.10	0.76	59	
	1½		7.6	68	5.15	0.59	45	
	2	6.7	7.5	63	4.70	0.57	43	
	2½		8.0	58	4.60	0.56	45	
	3	6.5	8.3	61	5.05	0.59	49	
	3½		8.9	56	4.95	0.56	50	
	4	6.5	7.9	57	4.50	0.55	44	
	4½		7.3	60	4.40	0.72	53	
	5	6.8	7.1	64	4.60	0.78	55	
	5½		7.0	57	3.95	0.76	53	
	6	6.3	7.3	65	4.75	0.72	53	

after it was constant or decreased gradually during the observation period, the final readings being one-half to one-third of the initial ones. Following the injection of bile salts, on the other hand, there was a marked increase in the total quantity excreted in the bile. Approximately 0.75 gm. was obtained in the second half hour specimen in Experiment 23 (Table V). This increase was for the most part confined to the first four collection periods after the injection. In the more satisfactory experiments sufficient bile acids to account for the whole of the amount injected were excreted during this 2 hour interval.

Similar results were obtained in the experiments on a dog with a permanent biliary fistula (Table VIII). Such animals consistently excrete a larger volume of bile than those operated on under anesthesia, but the concentration of bile acids is less. Apart from this difference, the physiologic response to the injection of bile salts was the same in both types of experiments. The recovery of the injected bile acids was slightly less complete in the animal with a permanent biliary fistula. However, qualitative tests showed the presence of bile acids in the urine and this renal loss will serve to explain the discrepancy.

A striking phenomenon in these experiments was the decrease in the intensity of the color of the bile during the periods of marked choleresis. The concentration of bilirubin in the bile during the preliminary periods varied from 54 to 186 mg. per cent. This concentration was promptly reduced to between 9 and 17 mg. following the injections, but the concentration gradually increased to a later maximum of from 56 to 147 mg. A consideration of the total quantity excreted shows that the normal rate of excretion was maintained during the 1st hour of the experiment, but thereafter, there was a gradual increase to 2 or 3 times that value between the 2nd and 5th hours. The excretion of bilirubin in the normal control was maintained at a relatively uniform rate throughout the experiment. The concentration in the bile increased slightly but the total quantity was constant apart from the fluctuations inherent in the collection and measurement of such small samples of bile.

Frerichs (7) observed the appearance of bilirubin in the urine after the intravenous injection of solutions of bile salts. He ascribed this to the transformation of bile salts into pigment. It

remained for Kühne (15) to point out that this extra pigment had its origin in hemoglobin set free in consequence of the hemolytic action of the bile acids. An additional control experiment (Table IX), was therefore performed in which the animal received 1 cc. of laked blood for each kilo of body weight. This injection of hemoglobin produced a threefold to fourfold rise in the excretion of bilirubin between the 2nd and the 6th hours. The maximal increase occurred about the 4th hour. There was no increase in the flow of bile in this experiment; the volume output was small, and the specimens were extremely concentrated.

TABLE IX.

Changes in Blood and Bile Following Intravenous Injection of Laked Blood.

Experiment 26. Weight of dog 12.0 kilos.

Time of collection.		Blood.	Bile.					Remarks.
Before injection.	After injection.	Bile acids.	Volume.	Bilirubin.		Bile acids.		
					Total.		Total.	
hr.	*min.*	*mg. per cent*	*cc.*	*mg. per cent*	*mg.*	*per cent*	*mg.*	
1			0.70	117	0.82	6.04	42	Control period.
½		5.1	0.60	186	1.11	7.70	46	
	5	4.2						12 cc. laked blood injected intravenously. Total bile acids 211 mg., total bilirubin 42.88 mg.
	10	5.1						
	15	4.4						
	30	5.0	0.50	264	1.32	6.91	35	
	45	5.1						
	hrs.							
	1	5.0	0.75	275	2.06	4.04	30	
	1½	5.1	0.45	670	3.01	4.22	19	
	2	5.7	0.45	625	2.82	4.00	18	
	2½		0.30	876	2.62	3.64	11	
	3		0.25	1000	2.50		10	
	3½		0.55	910	5.50	3.85	21	
	4	5.7	0.45	880	3.96	2.50	11	
	4½		0.65	880	5.70	2.86	19	
	5	5.1	0.50	710	3.55	2.50	13	
	5½		0.70	710	4.95	2.00	14	
	6	4.8	0.40	690	2.76	2.50	10	

Bilirubin alone produced a somewhat similar result. In Experiment 5 bilirubin was injected intravenously in a dosage equivalent to 2 mg. for each kilo of body weight (Table X). The pigment left the blood rapidly although at a slower rate than the bile salts. The serum bilirubin was increased to 2.5 mg. 5

TABLE X.

Changes in Blood and Bile Following Intravenous Injection of Bilirubin.

Experiment 27. Weight of dog 17.5 kilos.

Time of collection. Before injection.	Time of collection. After injection.	Blood. Bilirubin.	Bile. Volume.	Bile. Bilirubin.	Bile. Bilirubin. Total.	Bile. Bile acids.	Bile. Bile acids. Total.	Remarks.
hr.	*min.*	*mg. per cent*	*cc.*	*mg. per cent*	*mg.*	*per cent*	*mg.*	
1		0.0	0.70	35	0.25	3.35	23	Control period.
½		0.0	0.45	45	0.20	3.20	14	
	5	2.5						35 cc. solution containing 35 mg. bilirubin injected intravenously. Total bilirubin recovered 59.47 mg., total bile acids 615 mg.
	10	2.3						
	15	1.7						
	30	1.1	0.25	36	0.09	3.60	9	
	45	0.7						
	hrs.							
	1	0.6	0.60	120	0.72	6.55	40	
	1½	0.5	0.75	320	2.40	6.60	49	
	2	0.3	1.60	820	13.10	7.90	126	
	2½	0.2	1.80	1050	18.90	8.35	150	
	3	0.1	0.75	950	7.10	8.30	62	
	3½	0.0	0.45	840	3.80	8.40	38	
	4	0.0	0.45	700	3.15	7.40	33	
	4½	0.0	0.65	700	4.55	7.65	50	
	5	0.0	0.30	640	1.92	7.25	22	
	5½	0.0	0.30	645	1.94	6.10	18	
	6	0.0	0.30	600	1.80	6.10	18	

minutes after the injection. 2 hours later it had fallen to 0.3 mg. This curve of elimination from the blood is similar to curves reported in the first section of this paper. The maximal excretion in the bile came in the fourth, fifth, and sixth half hour periods after the injection. The bilirubin was injected in 0.1 per cent

solutions, but during these periods the bile contained approximately 1.0 per cent of bilirubin, a concentration 20 times that in the initial control specimens and 10 times that of the bile originally obtained from the gallbladder of the same dog. In this experiment, there was an increase in both the concentration and volume of the bile, hence there was a very great increase in the total excretion of pigment. The total quantity of bilirubin injected was recovered in the bile during the experimental period.

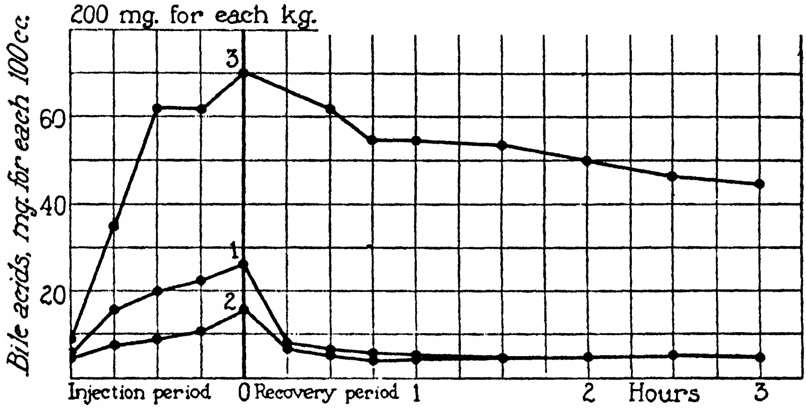

FIG. 1. Comparison of the changes in the bile acid content of the blood following the timed intravenous injection of (1) bile acids alone, (2) a mixture of bile acids and bilirubin, and (3) whole bile. The same dose of bile acids, 200 mg. for each kilo of body weight, was given in each experiment.

DISCUSSION.

The results obtained emphasize the independence in the physiologic behavior of bile acids and bilirubin. The increase in their concentration in the blood after the intravenous administration of bile or its constituents was determined by the dosage of each and the speed of injection. The bile acids are eliminated rapidly. Even the maximal dosage compatible with recovery of the animal, increases the concentration in the blood for only an hour or two. Injected bilirubin, on the other hand, leaves the blood at a fairly uniform rate over a period of several hours.

The experiments suggest that whole bile is more toxic than

either of its major constituents, a conclusion at variance with that of Horrall (12). Unfortunately there is no mention of dosage in his report so that it is difficult to compare the two series of experiments. The differences in the experimental technique may also serve to explain this discrepancy. We found that the injection of whole bile produced greater increases in the amount of bile acids in the blood, and the excess is more slowly removed from the blood stream, than when equivalent amounts are injected in pure solution (Fig. 1). Similarly, the injection of bilirubin solutions causes a lesser increase in the serum content and the excess is

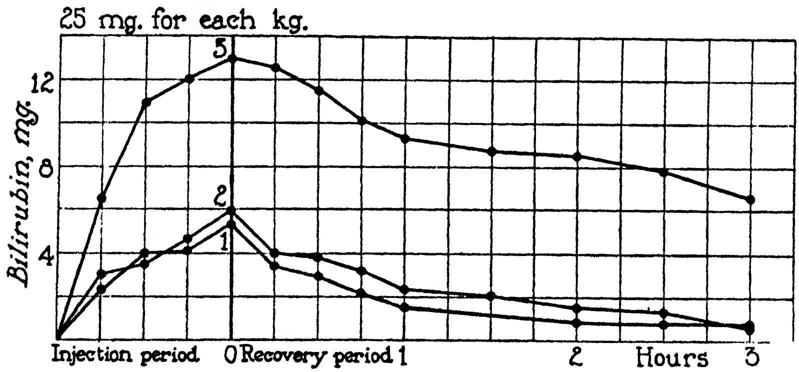

FIG. 2. Comparison of the changes in the serum bilirubin following the timed intravenous injection of (1) bilirubin alone, (2) a mixture of bilirubin and bile salts, and (3) whole bile. The same dose of bilirubin, 25 mg. for each kilo of body weight, was given in each experiment.

removed from the blood stream more rapidly than when bile containing an equivalent amount of pigment is given (Fig. 2). The elimination of these substances also may be greatly prolonged by various conditions which presumably affect the liver. This emphasizes the possibility of using injections of bile salts or bilirubin as tests for hepatic function. The clinical use of bilirubin has been reported by von Bergmann (3) and Eilbott (5), while studies on the elimination of the bile acids in experimental obstructive jaundice have been reported by Snell, Greene, and Rowntree (18). The curves showing the elimination of intravenously injected bile acids emphasize the rapidity of this process.

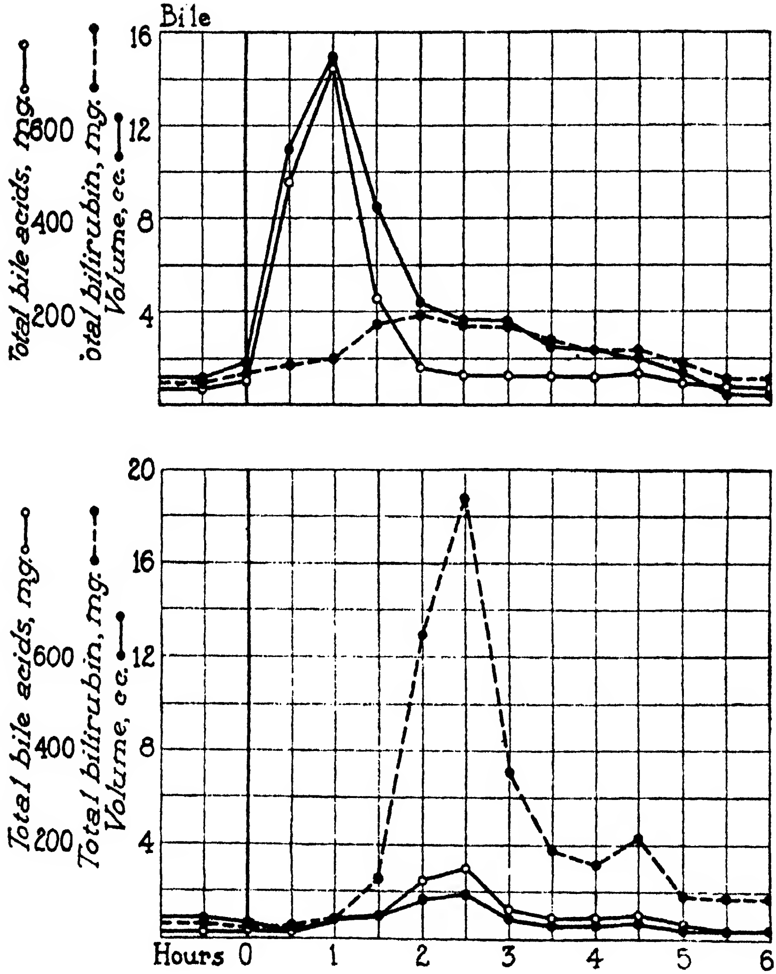

FIG. 3. Comparison of the changes in the volume and composition of the bile following the intravenous injection of bile acids and bilirubin. The upper chart shows the changes following the injection of bile acids in Experiment 23. The lower chart shows the changes following the injection of bilirubin in Experiment 27. The volume of the bile and the excretion of bile acids and bilirubin are shown. Two half hour collections of bile were taken in all experiments preceding the injection.

Their appearance in the bile follows the disappearance from the blood, with only a slight lag dependent on the rate of excretion by the liver. This depends in part on the condition of the animal; but if operative shock is avoided, or if an animal with a permanent biliary fistula is used, all of the injected bile acids may be recovered in the bile within 2 hours.

The cholagogue action of the bile acids is well shown in these experiments (Fig. 3). They are excreted primarily by an increase in the volume of the bile and the greatest change in the latter corresponded with the excretion of the greatest amount of the bile acids. The concentration of the bile acids was likewise maximal at such times, though the degree of change in concentration was much less marked than that in volume. The increase in the volume of bile, however, persisted for a period after the total output of the bile acids returned to the normal level. The bile in consequence was more dilute in the late after period.

The intravenous injection of solutions of bile salts results in the destruction of a certain number of erythrocytes. This is signified by an increase in the excretion of bilirubin during the later stages of the experiment. Similar changes in the output of pigment are produced by the intravenous injection of hemoglobin. When pure bilirubin is injected intravenously it also disappears from the blood to be excreted in the bile. This process, however, takes place more slowly than in the case of the bile acids, although seemingly it is equally complete. The excretion of injected bilirubin takes place primarily by an increase in the concentration of the bile rather than by a change in the volume output (Fig. 3). In one experiment there was an increase in the amount of bile during the periods of maximal excretion of bilirubin, but the cholagogue effect was slight, especially when contrasted with the marked effect of the bile acids. When the excretion of bilirubin is less pronounced, as after the injection of hemoglobin, the volume of the bile is not increased.

These experiments serve to emphasize the independence of the excretion of the bile acids and of bilirubin in the bile. The maximal concentration of bile acids observed was about 6 per cent. Foster, Hooper, and Whipple (6) reported concentrations of from 7 to 9 per cent in some of their experiments on animals

with fistulas. They used the gallbladder in the construction of the biliary fistula and a further concentration by this viscus cannot be excluded in their experiments. These high concentrations, however, were obtained in experiments in which the output of bile was reduced after the ingestion of sugar. We observed a concentration of 8 per cent of bile acids following the intravenous injection of bilirubin (Table X). In this experiment a relatively small amount of concentrated bile was obtained. The usual output was more dilute. The bile obtained in experiments on dogs with permanent biliary fistulas was in general more dilute than that secured by the direct intubation of the common duct under anesthesia. Injury to the liver by obstruction to the fistula or by infection further reduces the attainable concentration of bile acids in the bile. Apparently the usual range of variation in the concentration of bile acids in the bile as secreted by the liver is relatively slight and an increase in the output of bile acids is primarily attained through the medium of an increase in the volume of the bile. In this respect the condition is analogous to the condition in chronic nephritis in which there is a loss of the concentrating activity of the kidney, and the excretion of urea can be augmented only by an increase in the volume of the urine.

The liver is normally able to secrete bile of a widely varying bilirubin concentration. These experiments suggest that concentrations of from 20 to 1000 mg. for each 100 cc. are still within the limits of the normal. An increased excretion of bilirubin in the bile is brought about primarily by an increase in the concentration of the bile pigment, although when excessive amounts are present there may be an additional increase in the volume output. The data do not furnish very good evidence that bilirubin is washed out by an increased output of bile. On the other hand, the experiments were performed on normal animals in which bilirubin is excreted as rapidly as it is formed.

Clinical observations suggest that in some cases of disease of the liver, the bile contains only small amounts of pigment and "white bile" of hepatic origin may be present. Furthermore, although increased concentration of pigment in the bile is a recognized feature of hemolytic icterus this of itself is not sufficient to prevent the retention of bilirubin and the development of

jaundice. The importance of further study of the physiologic factors determining the excretion of bile acids and bilirubin under such pathologic conditions is obvious.

SUMMARY.

Whole bile, bilirubin, and the bile acids quickly disappear from the blood stream following their intravenous injection in dogs. The bile acids leave the blood rapidly. Even with maximal doses the excess is removed within 2 hours. Bilirubin, on the other hand, is eliminated at a somewhat slower rate. When whole bile, obtained at operation, is injected, there is a greater increase in the level of each constituent in the blood, and the excess leaves the blood less rapidly than if given in pure solution.

This study also presents the curves of excretion by the liver of intravenously injected bile acids and of bilirubin, and compares the simultaneous changes in the concentration in the bile with those in the blood.

Bile acids have a pronounced cholagogue action; it was greatest during the first 2 hours of these experiments but the increased flow of bile continued for a longer period. The intravenously injected bile acids were excreted primarily by an increase in the volume of the bile rather than by changes in its concentration. The total quantity of bile acids administered could be recovered in the bile within 2 hours.

Injections of bilirubin or hemoglobin increase the output of pigment in the bile. This augmented rate of excretion is brought about primarily by an increase in the concentration rather than by changes in the volume of the bile.

Excretion of injected bilirubin takes place more slowly than the excretion of the injected bile acids.

BIBLIOGRAPHY.

1. Aldrich, M., and Bledsoe, M. S., Studies in the metabolism of the bile. I. A quantitative Pettenkofer test applicable to the determination of bile acids in the blood, *J. Biol. Chem.*, 1928, lxxvii, 519.
2. van den Bergh, A. A. H., Der Gallenfarbstoff im Blute, Leiden, 1918, viii.
3. von Bergmann, G., Zur Funktionellen Pathologie der Leber insbesondere der Alkohol-ätiologie der Cirrhose, *Klin. Woch.*, 1927, vi, 776.

4. Brakefield, J. L., and Schmidt, C. L. A., Studies on the synthesis and elimination of certain bile components in obstructive jaundice, *J. Biol. Chem.*, 1926, lxvii, 523.
5. Eilbott, W., Funktionsprüfung der Leber mittels Bilirubinbelastung, *Z. klin. Med.*, 1927, cvi, 529.
6. Foster, M. G., Hooper, C. W., and Whipple, G. H., The metabolism of bile acids. III. Administration by stomach of bile, bile acids, taurine, and cholic acid to show the influence upon bile acid elimination, *J. Biol. Chem.*, 1919, xxxviii, 379.
7. Frerichs, F. T., A clinical treatise on diseases of the liver, London, 1860–61, i.
8. Greene, C. H., and Aldrich, M., Studies in the metabolism of the bile acids, *Am. J. Physiol.*, 1927, lxxxi, 480.
9. Greene, C. H., Aldrich, M., and Rowntree, L. G., Studies in the metabolism of the bile, *J. Physiol.*, 1927, lxiv, 7.
10. Greene, C. H., Snell, A. M., and Walters, W., Diseases of the liver. I. A survey of tests for hepatic function, *Arch. Int. Med.*, 1925, xxxvi, 248.
11. Hoppe-Seyler, F., Ueber die Anwesenheit von Gallensäuren im icterischen Harne und die Bildung des Gallenfarbstoffes, *Virchows Arch. path. Anat.*, 1862, xxiv, 1.
12. Horrall, O. H., The toxic substances in bile, *Proc. Inst. Med. Chicago*, 1927, vi, 254.
13. Huppert, H., Ueber das Schicksal des Gallensäuren im Icterus, *Arch. Heilk.*, 1864, v, 236.
14. Judd, E. S., Mentzer, S. H, and Parkhill, E., A bacteriological study of gallbladders removed at operation, *Am. J. Med. Sc.*, 1927, clxxiii, 16.
15. Kühne, W., Beiträge zur Lehre vom Icterus, *Virchows Arch. path. Anat.*, 1858, xiv, 310.
16. Rous, P., and McMaster, P. D., A method for the permanent sterile drainage of intraabdominal ducts, as applied to the common duct, *J. Exp. Med.*, 1923, xxxvii, 11.
17. Schiff, M., Gallenbildung abhängig von der Aufsaugung der Gallenstoffe, *Arch. ges. Physiol.*, 1870, iii, 598.
18. Snell, A. M., Greene, C. H., and Rowntree, L. G., Diseases of the liver. VII. Further studies in experimental obstructive jaundice, *Arch. Int. Med.*, 1927, xl, 471.

NOTE ON THE FRACTIONATION OF SERUM PROTEINS BY MEANS OF AMMONIUM SULFATE.

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We have adopted the method proposed by Van Slyke and Cullen (1) and modified by Petschacher, Berger, and Schretter (2), who removed the free ammonia of the albumin solution with a current of air instead of steam. We have further modified the method by aerating at 60–70°, and by the use of superoxol. Total nitrogen can be determined with sufficient accuracy in dilutions corresponding to 0.1 cc. of serum. Albumin plus non-protein nitrogen can be determined in dilutions corresponding to $\frac{1}{6}$ cc. of serum.

Total nitrogen is estimated in serum diluted tenfold with 0.9 per cent sodium chloride solution. 1 cc. of the dilution is digested with 2 cc. of concentrated sulfuric acid and 2.5 cc. of 2 per cent copper sulfate solution. (Practically the same figures for nitrogen are found if the oxidation is finished by addition of 0.2 cc. of superoxol, Merck.)

The globulin is precipitated by half saturation with ammonium sulfate in serum diluted threefold with distilled water. The ammonium sulfate is added under constant shaking and after short standing the mixture is centrifuged for about 30 minutes at a speed of 2500 R.P.M. The supernatant fluid is carefully pipetted and used for determination of albumin plus non-protein nitrogen. The ammonium sulfate is decomposed by magnesium oxide in 50 per cent alcohol, as suggested by Van Slyke. The liberated ammonia is carried off by a rather vigorous air current, washed free of ammonia, care being taken to push the aeration tube down as far as possible. The flasks are kept in a water bath of 60–70°. After 2 to 3 hours the ammonia is completely driven off. It is advisable to have the temperature of the water bath not higher than 70° to prevent the contents of the flasks

from drying out, for otherwise it becomes difficult to determine whether the ammonia has been removed completely. To the residue in the flasks a few cc. of distilled water, 2.5 cc. of 20 per cent copper sulfate, and 2 cc. of concentrated sulfuric acid are added, and the flasks are heated over a low flame until the contents appear to be in fine suspension; then the water is boiled off and as soon as white fumes begin to appear, 0.2 cc. of superoxol (Merck) are added; the mixture clears up after a few minutes.

TABLE I.

Solution used.	Calculated concentration, N per 100 cc.	No. of analyses.	Average concentration, N per 100 cc.	Deviation from average.	Deviations from calculated concentration.
	mg.		*mg.*	*per cent*	*mg. per 100 cc.*
Ammonium sulfate solution 2 cc. (1.508 mg. N).	75.42	3	75.17	+0.5, −0.1, −0.4	−0.25 (−0.3 per cent).
Urea solution 2 cc. (1.507 mg. N).	75.33	7	73.48	+0.7, +0.8, −0.8, −0.5, +0.4, +0.6, −0.4	−1.85 (−2.5 per cent).
Urea solution 25 cc. (18.83 mg. N).	75.33	4	74.12	+1, −1, +0.3, −0.3	−1.21 (−1.6 per cent).

The ammonium sulfate used was Merck's reagent quality, dried to constant weight at 105°. The urea used was Eimer and Amend's urea, pure, dried to constant weight over concentrated sulfuric acid. The estimations of urea were made on different days in duplicate or triplicate. Macro-Kjeldahl determinations were employed in the last four analyses.

(Without addition of superoxol the oxidation takes a few hours and needs much supervision.)

For determination of non-protein nitrogen the usual precipitation with tungstic acid is used.

We have used for distillation the apparatus suggested by Fuchs (3), with the modification of using a Hopkins trap and substituting a block tin coil and Pyrex tubing for the Jena glass tubing used by Fuchs.

To obviate repeated testing for the end-point of the distillation

it seems advisable to distil half the volume in the distilling flask over, starting with a total volume of about 40 cc. The distillation should be slow in the beginning and the tin coil should

TABLE II.

Protein Fractionation of Serum.

Sample No.	Total protein.	No. of determinations.	Deviation from average.	Albumin.	Globulin.	No. of determinations.	Deviation from average.	Ratio, globulin: albumin.
	gm. per 100 cc.		*per cent*	*gm. per 100 cc.*	*gm. per 100 cc.*		*per cent*	
1	7.12	4	+0.2, −0.5 +0.9, −0.5	4.80	2.33	3	−2, +0.8, +1.2	1:2.06
2	9.26	4	+0.1, +1.4 −0.3, −1.1	6.43	2.82	3	−0.4, +0.7 +0.1	1:2.27
3	8.07	4	−1.5, −0.2 +0.5, +1.2	5.40	2.67	4	−0.3, +0.2 +0.1, −0.2	1:2.02
4	7.65	4	+0.4, −1.9 +1.5, +0.1	5.13	2.51	4	+0.2, −2.3 +1.7, +0.5	1:2.04
5	7.78	4	−1.5, +2.6 −2.7, +1.6	5.22	2.56	4	+2.5, −0.8 −2, +0.5	1:2.04

The blood was taken from one person on different dates (December 27, 1927; January 10, January 30, February 8, February 22, 1928). It was not taken in the fasting state, but uniformity of conditions was maintained as much as possible. On January 10 (Sample 2) the blood became hemolyzed.

For determination of total nitrogen we have used a dilution corresponding to 0.1 cc. of serum. For determination of albumin plus non-protein nitrogen we have used a dilution corresponding to ⅛ cc. of serum. For determination of non-protein nitrogen we have used a dilution corresponding to 0.5 cc. of serum.

be cooled sufficiently. The excess acid is titrated with 0.05 N sodium hydroxide, with methyl red and methylene blue as indicators.

A micro burette of 5 cc. capacity, having subdivisions of 0.02 cc., is used.

The method gives satisfactory results on ammonium sulfate and urea, and also on serum, as shown in Tables I and II. It is also shown that for the same person the ratio globulin:albumin remains constant for a period of about 2 months, if the technique for obtaining blood is correct.

BIBLIOGRAPHY.

1. Cullen, G. E., and Van Slyke, D. D., *J. Biol. Chem.*, 1920, xli, 587.
2. Petschacher, L., Berger, W., and Schretter, G., *Z. ges. exp. Med.*, 1926, l, 449.
3. Fuchs, H., *Biochem. Z.*, 1926, clxxvi, 32.

CLINICAL CALORIMETRY.

XLII. A COMPARISON OF THE EFFECT OF GLUCOSE AND DIHYDROXYACETONE ON METABOLISM.

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INTRODUCTION.

The study of the intermediary metabolism of glucose has recently occupied the attention of a number of investigators who have approached the subject by using the triose dihydroxyacetone, $CH_2OH \cdot CO \cdot CH_2OH$. Rabinowitch (1) and Mason (2) have reviewed the earlier work with dihydroxyacetone which was limited on account of difficulties in its preparation. They noted in the course of their own investigations that blood sugar curves in normal subjects, following dihydroxyacetone administration, were only slightly elevated in comparison with those following an equal amount of glucose. Mason (2) found in normal men a more rapid and greater elevation of non-protein respiratory quotient, carbohydrate combustion, and total heat production after giving dihydroxyacetone than after giving glucose. In diabetic patients, Rabinowitch (1, 3) and Kermack, Lambie, and Slater (4) report improvement following the ingestion of dihydroxyacetone on the basis of a falling blood sugar level, reduction in the severity of ketosis, and improvement in general clinical appearance. Mason (5) reported in diabetic subjects a greater rise in the non-protein respiratory quotient, carbohydrate combustion, and heat production after dihydroxyacetone than after glucose. He noted at the same time that the rise in blood sugar curves was less marked and the return to fasting level was quicker after dihydroxyacetone. Himwich, Rose, and Maler (6) found a more rapid rise in the respiratory quotient of dogs after injecting dihydroxyacetone subcutaneously than after injecting the same amount of glucose.

Campbell and his collaborators studied the action of dihydroxyacetone

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in depancreatized dogs. They (7) noted no preferential utilization of dihydroxyacetone in these animals when it was compared with levulose, inulin, and glycerol. Campbell and Markowitz (8) reported increased oxygen consumption without elevation of the respiratory quotient, which was at the fat level. They discovered dihydroxyacetone present in the blood and noted a rise in the actual blood sugar which was coincident with the disappearance of dihydroxyacetone from the blood. They also found "extra glucose" in the urine equivalent to the amount of dihydroxyacetone ingested. They concluded that dihydroxyacetone was converted into glucose in the animal body, that insulin was not required for this conversion, and that it could not be metabolized directly by depancreatized animals. Markowitz and Campbell (9) observing animals after the liver, stomach, and intestines had been removed, reported that dihydroxyacetone injected intravenously was not converted into glucose but that it could be recovered from tissues and blood in sufficient quantities to account for the amounts given. They concluded that the conversion of dihydroxyacetone to glucose occurred in the liver. Campbell and Hepburn (10), Hewitt and Reeves (11), and Kermack, Lambie, and Slater (4) all found that dihydroxyacetone would relieve insulin hypoglycemia at approximately the same rate as glucose.

The studies with dihydroxyacetone presented in this paper were carried out in the calorimeter of the Russell Sage Institute in order to obtain the results of long period observations which can be made with this apparatus. Determinations were made on four normal and two diabetic men. In order to compare the metabolism of long periods as obtained in the calorimeter with the so called short period observation, five of these six subjects were studied with the Tissot apparatus thus giving in addition an opportunity for obtaining frequent samples of blood and urine.

Methods.

The respiration calorimeter of the Russell Sage Institute of Pathology was used to obtain the metabolic data for the long period observations. It has previously been described in Paper II of this series (12). No change in procedure was used except that the temperature of the air in the calorimeter was kept between 24.0° and 25.0°. One normal and two diabetic subjects were studied in the metabolism ward in connection with the calorimeter (13). The other three normal subjects were physicians who came to the laboratory without breakfast and rested for 1 to 2 hours before actual observations were started. The observations were

3 hours in length. Each subject was observed at least three times, once in basal conditions, once with glucose, and once with dihydroxyacetone. A thermos bottle with a small syphon was used so that the subject could easily take the solution, when inside of the calorimeter, after the preliminary period was completed. In basal observations the subjects were given 200 cc. of water, ½ grain of saccharin, and 0.5 gm. of citric acid making a palatable drink which was used as a control. In the other observations the saccharin was replaced by 50 gm. of glucose or by 50 gm. of dihydroxyacetone. The solution was placed in the thermos bottle at 38° so that it could be taken at approximately body temperature 30 to 45 minutes later when the observation was actually started. The urine was collected at the end of the observation. Nitrogen was determined by the Kjeldahl technique and sugar, if present, by Benedict's method (14).

In making the short period observations, the Tissot apparatus, a nose clip, mouthpiece, and 2-way valve, the Soderstrom model of the Lovên diaphragm valve (15), were used for the collection of expired air. The expired air was analyzed by a modified Henderson-Haldane gas analysis apparatus. The procedure with each subject was uniform and consisted of a basal period after at least 30 minutes rest, the ingestion of 50 gm. of either glucose or dihydroxyacetone, and observations taken at the end of 30 minutes, 1 hour, 2 hours, and 3 hours. The expired air was collected during the last 6 minutes of each period, a sample of blood taken at the end of each period, and a sample of urine voided at the end of each hour. Each sample of blood was tested for blood sugar by the method of Folin and Wu (16), for dihydroxyacetone by the method of Campbell (17), and in some observations for carbon dioxide-combining power by the method of Van Slyke and Cullen (18). Each sample of urine was tested for total nitrogen by the Kjeldahl method, for sugar, if present, by Benedict's method (14), and for dihydroxyacetone by the method of one of the authors (19).

As a control on the experimental data frequent alcohol checks were made on the calorimeter. The alcohol solution employed for these checks contained 92.07 per cent of alcohol by weight. The amount used per hour was between 9.7 and 11.5 cc. With calculations based on the chemical composition of the alcohol and

TABLE I.

Alcohol Checks for 1926–27.

Date.	Hour.	Error, per cent.				R. Q., theory 0.667.
		Heat.	O_2	CO_2	H_2O	
1926						
Oct. 20	1	+1.3	+0.7	+0.2	+17.0	0.663
	2	+0.3	−0.8	−1.5	+10.3	0.662
	3	+0.8	−1.9	−1.4	+10.3	0.671
Average...............		+1.0	−0.7	−0.9	+12.6	0.665
Oct. 28	1	+0.3	−1.6	−0.3	+6.0	0.676
	2	+0.9	−1.8	−1.0	+5.5	0.672
	3	+2.9	−1.3	+0.8	+6.5	0.681
Average...............		+1.4	−1.5	−0.1	+6.0	0.676
Nov. 4	1	−0.4	−2.6	−0.4	+3.8	0.681
	2	+0.8	−0.6	−0.5	+4.2	0.668
	3	−0.8	−1.8	−0.5	+3.0	0.675
Average...............		−0.1	−1.7	−0.5	+3.7	0.675
1927						
Jan. 4	1	−0.5	+0.2	−0.8	+4.2	0.660
	2	−0.8	−1.7	−0.4	+4.5	0.676
	3	±0.0	−1.6	−0.3	+2.2	0.675
Average...............		−0.4	−1.0	−0.5	+3.7	0.670
Feb. 3	1	−4.8	−3.8	−2.1	+2.2	0.679
	2	−1.3	−1.0	−1.8	+4.2	0.661
	3	−1.2	−2.3	−1.1	+1.9	0.674
Average...............		−2.5	−2.4	−1.7	+2.8	0.671
Feb. 15	1	−1.2	−3.8	−1.8	+3.4	0.681
	2	−0.5	−0.3	+0.4	+4.3	0.672
	3	−1.0	−0.7	−2.3	+1.6	0.656
Average...............		−0.9	−1.6	−1.2	+3.1	0.669
Mar. 1	1	−1.9	−2.8	−1.9	+3.4	0.673
	2	−1.0	−3.0	−3.4	+2.7	0.664
	3	−1.3	−2.4	−1.5	+3.0	0.673
Average...............		−1.4	−2.7	−2.3	+3.0	0.670
Mar. 17	1	+0.5	−1.4	−0.3	+4.2	0.674
	2	+1.2	−3.7	−0.6	+4.4	0.690
	3	+1.8	±0.0	−1.3	+5.8	0.658
Average...............		+1.2	−1.7	−0.7	+4.7	0.674

TABLE I—*Concluded.*

Date.	Hour.	Error, per cent.				R. Q., theory 0.667.
		Heat.	O_2	CO_2	H_2O	
1927						
Mar. 29	1	−0.5	−3.9	−1.4	+2.2	0.683
	2	+0.7	−1.1	+1.0	+4.8	0.681
	3	+0.8	−0.5	+1.3	+3.7	0.679
Average...............		+0.3	−1.9	+0.3	+3.3	0.680
Mar. 31	1	−1.2	−3.1	−0.6	+3.4	0.683
	2	−1.1	−1.3	−1.3	+3.6	0.666
Average...............		−1.1	−2.2	−1.0	+3.5	0.675
Apr. 5	1	−2.2	−0.2	−1.6	+4.4	0.657
	2	−0.5	−1.7	−1.6	+2.9	0.667
	3	−0.1	−0.6	−1.3	+4.0	0.662
Average...............		−0.9	−0.8	−1.5	+3.8	0.662
Apr. 28	1	−1.4	−1.0	−0.5	+5.3	0.670
	2	−0.8	+0.2	−1.8	+3.1	0.653
	3	−1.8	−1.0	−1.0	+3.6	0.666
Average...............		−1.3	−0.6	−1.1	+4.0	0.663
Total average.........		−0.4	−1.6	−0.9	+4.5	0.671

complete combustion assumed at an air temperature of 24.0°, 10 cc. of this solution give the following theoretical factors: O_2 15.53 gm., CO_2 14.23 gm., H_2O 9.38 gm., and heat 52.73 calories. The results of all the alcohol checks are presented in Table I. The gas analysis apparatus was tested by frequent outdoor air analyses. All chemical determinations were carried out in duplicate. A trained subject in basal condition was studied with the Tissot apparatus and then placed in the calorimeter for further basal observations.

PROTOCOLS.

Subject 1, E. M., Normal.—A laborer, age 39, Russian, was admitted to Bellevue Hospital January 15, 1927, complaining of weakness and vague pains in the left side of the body. For 10 months, since a slight accident, he noticed discomfort throughout the left side of the body and more pain in the left chest.

Physical examination showed an apathetic complaining man with some hyperesthesia over the left chest but otherwise there were no abnormal findings.

Laboratory tests revealed normal blood and urine, Wassermann test negative, normal electrocardiogram, and x-ray of heart and lungs normal.

During his stay in the hospital there were periods when he seemed somewhat depressed but no psychosis developed. He was studied as a normal control with four calorimeter and two Tissot observations. In the first

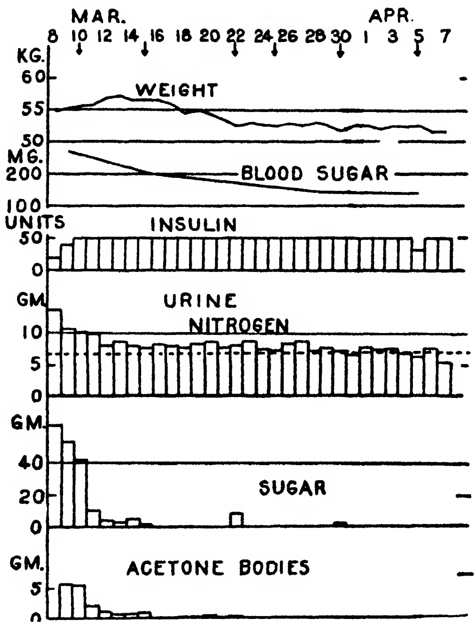

CHART I. Subject C. W.; laboratory findings shown graphically. The broken line signifies the nitrogen in the food; the arrows, the points at which the metabolism was observed.

calorimeter observation his temperature by rectum was 38.5° so it has been excluded from the comparisons.

Subject 2, A. B., Normal.—A physician, age 24, Argentinian, who was in good health. He was studied with four calorimeter and two Tissot observations.

Subject 3, H. J. D., Normal.—A physician, age 29, American, who was in good health. He was studied with three calorimeter observations.

Subject 4, W. S. M., Normal.—A physician, age 31, American, who was in good health. He was studied with three calorimeter and two Tissot observations.

Subject 5, C. W., Diabetes Mellitus.—A baker, age 29, Austrian, was admitted to Bellevue Hospital March 5, 1927, complaining of polydipsia, polyuria, and loss of weight. His history of diabetes dated back 1 year during which he had been on a moderately restricted diet at home. The symptoms noted had appeared during the past month.

Physical examination showed a thin young man in no pain, with some poor teeth, moderately large tonsils, scars from burns on left arm, and otherwise normal.

He had a normal temperature, pulse, and respiration. The remaining laboratory data with daily weights and insulin used are presented in Chart I. He received a diet of 1800 calories made up of protein 42 gm., fat 148

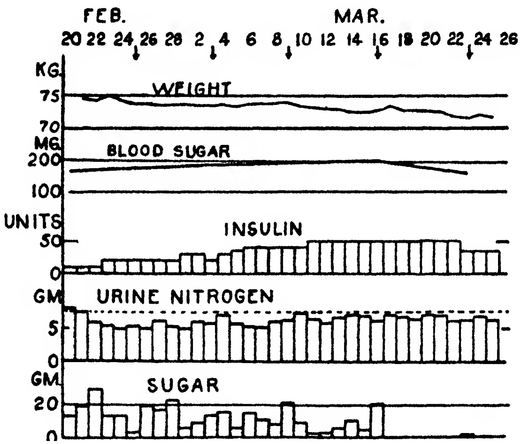

CHART II. Subject J. M.; laboratory findings shown graphically. The broken line signifies the nitrogen in the food; the arrows, the points at which the metabolism was observed.

gm., and carbohydrate 62 gm. until March 27, when it was increased to 2000 calories with protein 42 gm., fat 165 gm., and carbohydrate 71 gm. His condition improved rapidly with regulation of diet and insulin. He showed a mild ketosis on admission. Four calorimeter and two Tissot observations were made. The first calorimeter observation was made with dihydroxyacetone while he was still in a mild ketosis. The other five were made after his condition had improved.

Subject 6, J. M., Diabetes Mellitus.—A salesman, age 43, American, was admitted to the Neurological service of Bellevue Hospital February 7, 1927, complaining of loss of vision and pain in the legs. He had had severe burns from gasoline, in 1911, and gonorrhea, in 1918. His disturbance of vision was first noted 6 months ago and since then it has progressed so that he can only recognize large objects and has difficulty in finding his way

about the city. For a year he has noted sharp pains in both legs and weakness of the knees when climbing stairs. There has been some pain in the back, mostly in the shoulder and upper lumbar regions. He has lost 100 pounds in the past 2 years.

Physical examination revealed a moderately obese man with loose dry skin. Pupils reacted to light and accommodation. Fundi and external muscles of eye were normal. Heart sounds were distant. Blood vessels were palpable but not beaded, lower extremities were cold. Knee jerks were present but very sluggish.

Laboratory findings showed temperature 36–38°, pulse 60 to 80, respiration 16 to 24, blood and spinal fluid Wassermann tests negative, and urine with 1 to 2 per cent sugar and a trace of acetone.

A diagnosis of diabetes was made and the patient transferred to the Medical service. His case proved to be somewhat resistant to treatment and symptoms were very slow to improve. While under observation a severe pain developed in the right side of his face and x-ray revealed a cloudy sinus. It was drained and foul pus obtained followed by relief of pain and general improvement in his condition. He received a diet of 2000 calories made up of protein 48 gm., fat 164 gm., and carbohydrate 68 gm. Chart II contains the data of weight, insulin units, and blood and urine examinations during the period of actual observations. He was studied with three calorimeter and four Tissot observations all of which, except the final basal series with the Tissot apparatus, were made during the period before his sinus showed the signs of infection.

Subject 7, E. F. D. B., Normal.—A physician, age 44, American, who was in good health and whose metabolism has been studied frequently for the past 14 years (20). His basal metabolism was determined by both Tissot apparatus and calorimeter on the same day. No observations with either glucose or dihydroxyacetone were made.

Comment on Experimental Data.

Table II contains the complete data of all calorimeter observations. The 1st hour, recorded in the time column, is the end of the preliminary period which represents the actual start of the observation. All periods were 1 hour in length except as noted in the table in the case of A.B. and W.S.M., where on two occasions the period was 2 hours in length. The marked increase in oxygen used by W.S.M. in the observation with dihydroxyacetone may partly be due to a mild headache causing some extra movements during the early part of the experiment. The per cent of calories per hour from each type of food metabolized is based on the amount of nitrogen in the urine, which multiplied by 26.51 gives the calories from protein and on the table of Zuntz and Schumburg

as modified by Lusk (21) which gives the partition of the non-protein calories between fat and carbohydrate. The carbohydrate in gm. represents the amount of carbohydrate metabolized per hour on this basis.

Table III presents the complete data of the Tissot observations. The figures represent data for 1 hour periods as calculated from that obtained in a 6 minute observation. We can give no explanation for the marked elevation of carbon dioxide in the last period of the observation of C. W. with glucose. The columns under blood and urine headed "R. S." represent the total amount of M-substances of which the composition is not known and which will reduce the phosphomolybdate solution used in their determination. To determine the amounts of dihydroxyacetone present, the values obtained for basal blood and urine are taken as blanks and deducted from the values obtained in the other samples. The slight differences found were not tabulated as dihydroxyacetone in blood because they were considered to be within the range of error of the method used.

Charts III and IV are graphic presentations of carbon dioxide elimination, oxygen consumption, respiratory quotient, and heat production of the subjects E.M. and C.W.

DISCUSSION.

Dihydroxyacetone, taken by mouth, caused an increased output of carbon dioxide during the 1st hour as determined by both Tissot and calorimeter methods. It reached a very high level at the end of 30 minutes as noted by the Tissot studies. There was also an increased oxygen consumption which was most marked within the 1st hour. A rapid rise of the respiratory quotient occurred during that same period. The maximum quotient obtained in the calorimeter was 0.99 and with the Tissot method 1.09. With two subjects in the calorimeter the highest quotient occurred during the 2nd hour. An elevation of the respiratory quotient is produced either by an increased carbon dioxide elimination or by a decreased oxygen absorption. The increase noted after dihydroxyacetone was due to a marked increase in carbon dioxide elimination, as there was no decrease in oxygen absorption.

The cause of the marked increase in carbon dioxide elimination

TABLE II.

Calorimeter Data.

Subject, date (1927), weight, and surface area.	End of period.	CO_2	O_2	R. Q.	H_2O	Urine N per hr.	Non-protein R. Q.	Indirect calorimetry.	Heat eliminated.	Direct calorimetry.	Rectal temperature.	Pulse rate.	Work adder.	Per cent calories from: Protein.	Fat.	Carbohydrate.	Carbohydrate per hr.	Remarks.
		gm.	*gm.*		*gm.*	*gm.*		*calories*	*calories*	*calories*	*°C.*		*cm.*				*gm.*	
E. M., Jan. 21,	12.05										38.31							Temperature elevated.
68.4 kg., 1.79	1.05	25.2	23.5	0.78	34.2	0.49	0.77	77.6	75.4	81.9	38.43			17	64	19	3.7	
sq. m.	2.05	25.6	23.0	0.81	33.7	0.49	0.81	76.5	77.4	78.2	38.45			17	52	31	5.7	
(Normal.)	3.05	25.7	24.2	0.77	34.5	0.49	0.77	79.7	80.0	81.2	38.48			17	64	19	3.8	
E. M., Jan. 26,	11.48										37.02							Basal.
68.0 kg., 1.78	12.48	21.0	17.8	0.88	26.0	0.24	0.87	60.4	63.9	64.1	37.03		28	10	38	52	7.6	
sq. m.	1.48	21.8	19.3	0.82	27.2	0.24	0.83	64.6	65.4	67.9	37.08			10	50	40	6.2	
	2.48	21.9	19.6	0.81	28.0	0.24	0.81	65.7	66.1	66.9	37.10			10	57	33	5.4	
E. M., Feb. 4, 67.8	11.47										37.18							Glucose 50 gm. at 11.03 a.m.
kg., 1.78 sq. m.	12.47	23.2	18.6	0.91	33.1	0.33	0.93	63.6	67.7	59.5	37.04		4	14	20	66	10.2	
	1.47	22.4	18.1	0.90	30.9	0.33	0.92	61.7	66.2	67.6	37.07		10	14	23	63	9.5	
	2.47	22.2	17.2	0.94	29.9	0.33	0.97	59.3	65.2	60.4	36.99		13	15	8	77	11.1	
E. M., Jan. 28,	11.42										37.11							Dihydroxyacetone 50 gm. at 11.04 a.m.
67.9 kg., 1.78	12.42	23.6	18.2	0.95	27.7	0.30	0.97	62.7	66.0	61.7	37.04		7	12	9	79	12.1	
sq. m.	1.42	22.8	18.5	0.90	28.2	0.30	0.91	63.1	68.3	68.5	37.05		4	12	25	63	9.5	
	2.42	21.5	19.0	0.82	28.2	0.30	0.82	63.7	69.2	62.1	36.93		6	12	53	35	5.4	

A. B., Feb. 2, 56.3	11.37										37.22	66						Basal.
kg., 1.61 sq. m.	12.37										37.12	72	26					
(Normal.)	1.37	41.1*	33.1	0.90	56.5	0.69	0.96	111.5	131.5	125.7	37.11	68	12	33	9	58	8.0	
	2.37	20.7	16.9	0.89	26.3	0.69	0.94	56.8	63.6	67.0	37.19	68	8	32	13	55	7.6	
A. B., Mar. 4, 57.2	11.30										36.96							Saccharin ½ gr. at
kg., 1.62 sq. m.	12.30	20.5	16.8	0.89	21.7	0.62	0.93	56.5	57.5	63.0	37.08	68	10	29	16	55	7.6	11.33 a.m.
	1.30	19.8	16.9	0.85	22.3	0.62	0.87	56.5	59.6	57.5	37.04	65	10	29	30	41	5.6	
	2.30	20.6	17.3	0.86	22.7	0.62	0.89	58.0	57.0	59.6	37.10	64		28	26	46	6.5	
A. B., Feb. 23,	12.00										37.06							Glucose 50 gm. at
56.9 kg., 1.61	1.00	23.2	18.0	0.94	28.4	0.59	0.99	61.4	62.9	64.5	37.10	72	12	25	2	73	10.9	12.03 p.m.
sq. m.	2.00	23.3	17.6	0.96	27.2	0.59	1.02	60.5	63.3	61.1	37.06	66	10	26	0	74	11.0	
	3.00	22.3	17.1	0.95	25.9	0.59	1.01	58.4	60.8	59.6	37.04	68	12	27	0	73	10.5	
A. B., Feb. 18,	11.42										37.04							Dihydroxyace-
56.5 kg., 1.61	12.42	23.7	17.4	0.99	31.0	0.78	1.10	59.4	60.7	64.1	37.12	68	24	35	0	65	9.3	tone 50 gm. at
sq. m.	1.42	20.4	16.0	0.93	28.2	0.78	1.02	53.8	60.1	62.1	37.17	62	4	38	0	62	8.1	11.48 a.m.
	2.42	24.4	18.6	0.95	28.9	0.78	1.04	63.4	61.0	65.4	37.27	68		33	0	67	10.4	
H. J. D., Mar. 11,	11.21										37.29							Saccharin ½ gr. at
85.5 kg., 2.00	12.21	23.8	20.5	0.85	30.1	0.40	0.86	68.8	73.2	60.2	37.11		4	15	40	45	7.6	11.25 a.m.
sq. m.	1.21	23.5	20.1	0.85	27.2	0.40	0.86	67.8	72.4	59.3	36.93		3	16	38	46	7.6	
(Normal.)	2.21	22.4	20.2	0.80	26.2	0.40	0.80	67.3	73.1	66.4	36.84		4	16	57	27	4.5	
H. J. D., Apr. 12,	11.35										37.09							Glucose 50 gm.
86.2 kg., 2.00	12.35	24.9	21.6	0.84	27.4	0.38†	0.84	72.5	69.9	58.9	36.94	56	6	14	45	41	7.2	at 11.39 a.m.
sq. m.	1.35	24.9	20.4	0.89	26.6	0.38	0.90	69.3	76.6	59.8	36.71	58	3	15	27	58	9.8	
	2.35	28.0	21.2	0.96	28.2	0.38	0.99	73.4	73.0	79.2	36.80	58	16	14	3	83	14.9	

* 2 hour period.

† Average of two other determinations.

TABLE II—*Concluded.*

Subject, date (1927), weight, and surface area.	End of period.	CO_2	O_2	R. Q.	H_2O	Urine N per hr.	Non-protein R. Q.	Indirect calorimetry.	Heat eliminated.	Direct calorimetry.	Rectal temperature.	Pulse rate.	Work adder.	Per cent calories from: Protein.	Fat.	Carbohydrate.	Carbohydrate per hr.	Remarks.
		gm.	*gm.*		*gm.*	*gm.*		*calories*	*calories*	*calories*	*°C.*		*cm.*				*gm.*	
H. J. D., Mar. 18,	11.42										36.97							Dihydroxyace-
85.3 kg., 2.00	12.42	27.1	22.0	0.90	34.7	0.36	0.91	75.1	74.5	59.3	36.76		5	13	25	62	11.4	tone 50 gm. at
sq. m.	1.42	28.8	21.3	0.98	32.0	0.36	1.01	74.3	76.5	76.9	36.77		3	13	0	87	15.9	11.47 a.m.
	2.42	22.1	19.3	0.83	29.6	0.36	0.84	64.8	74.0	58.2	36.55		5	15	45	40	6.3	
W. S. M., Mar. 24,	11.52										37.02							Saccharin ½ gr. at
78.0 kg., 1.93	12.52	24.1	21.5	0.82	31.0	0.43	0.82	71.8	73.0	67.5	36.94	76	17	16	50	34	5.9	11.56 a.m.
sq. m.	1.52				31.0				72.1	60.8	36.77	72	16					
(Normal.)	2.52	50.8*	44.0	0.84	34.1	0.43	0.85	147.8	76.4	85.8	36.92	70	12	16	41	43	7.8	
W. S. M., Apr. 19,	11.38										37.19							Glucose 50 gm. at
78.8 kg., 1.93	12.38	26.3	23.0	0.83	39.9	0.45	0.84	76.9	81.2	71.0	37.04		14	16	44	40	7.6	11.42 a.m.
sq. m.	1.38	26.4	21.8	0.88	37.2	0.45	0.90	73.8	81.2	80.2	37.03		14	16	27	57	10.3	
	2.38	27.2	21.4	0.92	38.2	0.45	0.95	73.4	80.7	71.8	36.90		17	16	13	71	12.7	
W. S. M., Apr. 14,	11.32										36.96	80						Dihydroxyace-
79.1 kg., 1.93	12.32	30.3	25.7	0.86	38.5	0.64	0.87	86.4	83.3	79.7	36.91	76	21	20	35	45	9.5	tone 50 gm. at
sq. m.	1.32	31.1	24.9	0.91	39.6	0.64	0.94	84.7	85.3	79.7	36.83	76	19	20	15	65	13.4	11.36 a.m. Mild
	2.32	25.3	21.6	0.85	36.8	0.64	0.87	72.3	79.7	78.6	36.82	72	14	24	32	44	7.8	headache.
C. W., Mar. 15,	11.57										36.59							Saccharin ½ gr.
56.3 kg., 1.63	12.57	18.2	16.9	0.78	27.6	0.25	0.78	56.0	61.0	52.9	36.42		4	11	66	23	3.2	at 12.00 n.
sq. m.	1.57	17.2	16.1	0.78	24.1	0.25	0.78	53.3	58.4	55.3	36.36		2	12	66	22	2.9	
(Diabetic.)	2.57	17.5	16.6	0.77	23.4	0.25	0.76	54.9	56.5	47.5	36.17			12	71	17	2.2	

C. W., Mar. 22, 53.5 kg., 1.60 sq. m.	12.15										36.42							Glucose 50 gm. at 12.18 p.m.
	1.15	17.3	15.8	0.79	23.6	0.34	0.79	52.5	58.9	51.6	36.26		16	17	58	25	3.2	
	2.15	17.1	15.8	0.79	22.5	0.34	0.78	52.4	55.9	52.2	36.18		13	17	61	22	2.8	
	3.15	17.5	15.6	0.82	21.4	0.34	0.82	51.9	55.1	48.8	36.04			17	50	33	4.1	
C. W., Mar. 10, 54.9 kg., 1.61 sq. m.	11.42										36.34							Dihydroxyacetone 50 gm. at 11.45 a.m. Patient in mild acidosis.
	12.42	23.8	21.2	0.82	23.7	0.46	0.82	70.8	62.9	60.0	36.28		7	17	50	33	5.7	
	1.42	19.4	19.9	0.71	22.8	0.46	0.69	64.6	62.1	60.1	36.24		3	19	81	0	0	
	2.42	18.3	18.0	0.74	22.5	0.46	0.72	58.6	64.0	58.8	36.13		7	21	75	4	0.6	
C. W., Mar. 25, 52.5 kg., 1.59 sq. m.	11.51										36.51							Dihydroxyacetone 50 gm. at 11.53 a.m. No acidosis.
	12.51	21.9	16.7	0.95	23.8	0.27	0.98	57.9	53.6	48.6	36.40		10	12	5	83	11.5	
	1.51	18.5	17.2	0.78	23.1	0.27	0.78	57.0	58.9	52.6	36.26		8	12	65	23	3.2	
	2.51	17.0	16.5	0.75	21.6	0.27	0.74	54.2	53.2	48.6	36.16		9	13	77	10	1.3	
J. M., Feb. 25, 74.1 kg., 1.86 sq. m. (Diabetic.)	11.51										36.91							Saccharin ¼ gr. at 11.57 a.m.
	12.51	20.3	19.2	0.77	26.4	0.21	0.77	63.5	65.2	61.2	36.85			9	71	20	3.2	
	1.51	19.0	18.0	0.77	24.9	0.21	0.76	59.5	65.9	68.1	36.89			9	74	17	2.4	
	2.51	19.1	18.1	0.77	24.1	0.21	0.76	60.1	65.7	64.3	36.87			9	73	18	2.7	
J. M., Mar. 9, 72.2 kg., 1.84 sq. m.	11.32										37.01							Glucose 50 gm. at 11.35 a.m.
	12.32	21.6	20.4	0.77	27.6	0.33	0.76	67.5	73.6	70.3	36.96		17	13	71	16	2.7	
	1.32	20.9	20.1	0.76	28.3	0.33	0.75	66.1	73.6	63.1	36.79		17	13	73	14	2.2	
	2.32	19.9	19.1	0.76	26.6	0.33	0.75	62.9	70.1	65.6	36.72		7	14	72	14	2.2	
J. M., Mar. 3, 72.7 kg., 1.84 sq. m.	12.35										36.82							Dihydroxyacetone 50 gm. at 12.39 p.m.
	1.35	21.8	20.0	0.79	23.5	0.25	0.79	66.6	68.5	68.8	36.83		28	10	63	27	4.4	
	2.35	21.0	19.4	0.79	23.4	0.25	0.79	64.5	69.4	66.8	36.79		13	10	64	26	4.1	
	3.35	22.0	20.2	0.79	24.1	0.25	0.79	67.2	66.5	62.0	36.72		9	10	63	27	4.4	

TABLE III.

Tissot Data.

Subject, date (1927), and surface area.	Time.	CO_2	O_2	R.Q.	Urine N per hr.	Non-protein R.Q.	Calories per hr.	Pulse rate.	Respiratory rate.	Per cent calories from: Protein.	Fat.	Carbohydrate.	Carbohydrate per hr.	Blood. Sugar.	R.S.*	CO_2	Urine. Sugar.	R.S.*	Remarks.
	min.	*gm.*	*gm.*		*gm.*								*gm.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*	*gm. per hr.*	*mg. per hr.*	
E. M., Feb. 1, 1.78 sq. m.	Basal.	20.1	16.8	0.87	0.46	0.89	56.6	58	16	22	28	50	7.0	87	13		0	29	Normal; glucose 50 gm.
	30	22.4	19.7	0.83	0.49	0.83	65.7	60	13	20	45	35	5.6	109	15		0	36	
	60	25.2	20.0	0.92	0.49	0.95	68.3	58	14	19	13	68	11.4	141	14		0	36	
	120	22.1	18.8	0.86	0.43	0.87	63.3	60	13	18	35	47	7.3	133	12		0	29	
	180	21.7	17.9	0.89	0.43	0.91	60.3	58	18	19	23	58	8.5	95	11		0	29	
E. M., Feb. 9.	Basal.	21.7	19.5	0.81	0.41	0.81	65.0	61	16	17	52	31	4.9	91	15		0	28	Dihydroxyacetone 50 gm.
	30	31.1	22.7	1.00	0.50	1.04	79.0	65	17	17	0	83	16.1	97	17		0	70	
	60	23.3	21.2	0.80	0.50	0.80	70.3	68	16	19	54	27	4.6	80	17		0	70	
	120	24.0	19.8	0.88	0.48	0.90	67.1	60	17	19	26	55	9.0	89	18		0	95	
	180	22.8	20.4	0.81	0.37	0.82	68.0	62	16	14	51	35	5.7	89	15		0	87	
A. B., Apr. 29, 1.61 sq. m.	Basal.	18.8	17.4	0.79	0.35	0.78	57.6	64	12	16	62	22	3.0	93	11		0	10	Normal; glucose 50 gm.
	30	21.2	18.8	0.82	0.73	0.83	62.2	68	14	31	39	30	4.6	130	11		0	6	
	60	23.7	19.4	0.89	0.73	0.93	65.2	66	12	30	16	54	8.7	133	11		0	6	
	120	20.8	17.2	0.88	0.56	0.90	57.9	66	12	26	24	50	7.1	95	12		0	5	
	180	19.8	17.1	0.84	0.56	0.86	57.2	68	12	26	34	40	5.6	78	11		0	5	

A. B., Apr. 22.	Basal.	19.3	17.1	0.82	0.23	0.82	57.3	66	12	11	54	35	5.0	93	10	59	0	7	Dihydroxyacetone 50 gm.
	30	29.1	19.7	1.07	0.53	1.15	69.7	62	16	20	0	80	13.4	98	10	60	0	19	
	60	21.7	19.3	0.82	0.53	0.82	64.1	70	14	22	47	31	4.9	63	10	60	0	19	
	120	21.3	17.1	0.90	0.48	0.94	58.1	68	13	22	15	63	8.9	107	10	64	0	41	
	180	19.6	18.2	0.78	0.58	0.78	59.7	68	14	26	55	19	2.8	99	10	63	0	45	
W. S. M., Jan. 27, 1.93 sq. m.	Basal.	26.7	24.7	0.79	0.54	0.78	81.5	68	16	18	61	21	4.3	95	14		0	20	Normal; glucose 50 gm.
	30	29.9	27.0	0.81	0.58	0.81	89.8	64	15	17	52	31	6.7	156	14		0	30	
	60	29.1	26.0	0.82	0.58	0.82	86.5	70	17	18	49	33	6.9	145	14		0	30	
	120	24.6	21.6	0.83	0.51	0.84	72.2	70	16	19	43	38	6.7	105	16		0	25	
	180	25.0	22.0	0.83	0.35	0.83	73.6	62	17	13	49	38	6.8	85	18		0	17	
W. S. M., Feb. 11.	Basal.	26.3	22.9	0.84	0.57	0.85	76.5	64	13	20	39	41	7.6	102	12		0	15	Dihydroxyacetone 50 gm.
	30	38.7	25.9	1.09	0.64	1.16	91.8	71	16	20	0	80	17.6	92	11		0	87	
	60	28.1	25.0	0.82	0.64	0.82	83.0	75	16	21	48	31	6.3	66	13		0	87	
	120	26.1	22.5	0.84	0.59	0.85	75.2	62	17	21	39	40	7.3	86	16		0	99	
	180	24.9	22.3	0.81	0.60	0.82	73.9	62	14	21	48	31	5.6	90	16		0	76	
C. W., Mar. 30, 1.61 sq. m.	Basal.	20.9	16.5	0.92	0.13	0.93	56.9	54	9	6	22	72	10.0	111	11	46	0	3	Diabetic; glucose 50 gm.
	30	18.8	16.9	0.81	0.36	0.81	56.3	58	9	17	53	30	4.1	217	11	60	0	17	
	60	20.1	17.3	0.85	0.36	0.86	58.1	58	10	16	39	45	6.3	280	13	56	0	17	
	120	19.2	17.4	0.80	0.34	0.80	57.8	56	10	16	56	28	3.9	333	11	54	1.0	21	
	180	23.7	17.5	0.98	0.40	1.03	60.8	56	11	17	0	83	12.2	274	13	55	1.1	26	
C. W., Apr. 5.	Basal.	17.8	16.7	0.78	0.19	0.77	55.3	56	11	9	70	21	2.8	137	8	61	0	6	Dihydroxyacetone 50 gm.
	30	22.1	17.2	0.94	0.22	0.95	59.2	58	11	10	15	75	10.8	172	9	59	0	17	
	60	19.4	16.8	0.84	0.22	0.84	56.7	62	11	10	48	42	5.9	200	11	53	0	17	
	120	16.3	16.7	0.71	0.35	0.69	54.4	64	11	17	83	0	0	211	10	57	0	84	
	180	17.0	16.7	0.74	0.31	0.73	54.7	62	11	15	79	6	0.9	181	9	62	Trace.	89	

* This column represents the M-substances which are those that will reduce acid phosphomolybdate solutions.

I—Co

Subject, date (1927), and surface area.	Time.	CO_2	O_2	R.Q.	Urine N per hr.	Non-protein R.Q.	Calories per hr.	Pulse rate.	Respiratory rate.	Per cent calories from: Protein.	Fat.	Carbohydrate.	Carbohydrate per hr.	Blood. Sugar.	R.S.*	CO_2	Urine. Sugar.	R.S.*	Remarks.
	min.	gm.	gm.		gm.								gm.	mg. per 100 cc.	mg. per 100 cc.	per cent	gm. per hr.	mg. per hr.	
J. M., Mar. 16, 1.84 sq. m.	Basal.	19.5	20.8	0.68	0.22	0.67	68.1	56	17	9	91	0	0	199	12	57	0	16	Diabetic; glucose 50 gm.
	30	21.6	23.5	0.67	0.27	0.66	76.8	67	17	10	90	0	0	282	12	44	2.1	27	
	60	21.8	23.1	0.69	0.27	0.67	75.6	68	17	10	90	0	0	336	12	53	2.1	27	
	120	23.0	22.8	0.73	0.38	0.72	74.6	58	17	13	83	4	0.7	331	12	55	7.3	73	
	180	21.2	21.6	0.71	0.34	0.70	70.6	58	16	13	87	0	0	292	13	56	5.7	57	
J. M., Mar. 23.	Basal.	20.6	20.2	0.74	0.18	0.74	66.6	58	16	7	82	11	1.8	154	9	54	0	14	Dihydroxyacetone 50 gm.
	30	19.5	20.3	0.70	0.20	0.69	66.2	56	17	8	92	0	0	159	10	57	0	26	
	60	20.9	19.2	0.79	0.20	0.79	64.0	62	17	8	64	28	4.3	181	11	54	0	26	
	120	21.7	19.4	0.81	0.25	0.81	64.9	62	16	10	57	33	5.1	224	11	58	0	74	
	180	19.4	21.6	0.66	0.24	0.64	70.4	64	18	9	91	0	0	234	11	59	0.3	108	
J. M., Mar. 28.	Basal.	19.6	20.4	0.70			66.7	54	16										
J. M., May 10.	Basal.	18.4	18.1	0.74	0.27	0.73	59.2	66	20	12	81	7	1.0						All observations basal without removing mouth piece or nose clip.
	25	17.7	17.4	0.74	0.27	0.73	56.9	68	19	13	80	7	1.0						
	45	18.4	18.0	0.75	0.27	0.74	59.0	66	18	12	77	11	1.6						
	65	19.7	19.0	0.76	0.27	0.75	62.4	70	18	12	74	14	2.2						

E. F. D. B., Apr. 7, 1.95 sq. m.	Tissot. Basal.	22.4	20.7	0.79	0.59	0.78	68.2	50	10	23	57	20	3.3						Comparison of metabolism determined by Tissot method and calorimeter.
	Calorimeter. 60	22.8	20.7	0.80	0.53	0.80	68.6	50		21	53	26	4.4						
	120	23.4	21.7	0.79	0.53	0.78	71.5	49		20	58	21	3.6						

is not known. It may come from increased oxidation of carbohydrate in the body or from the conversion of carbohydrate

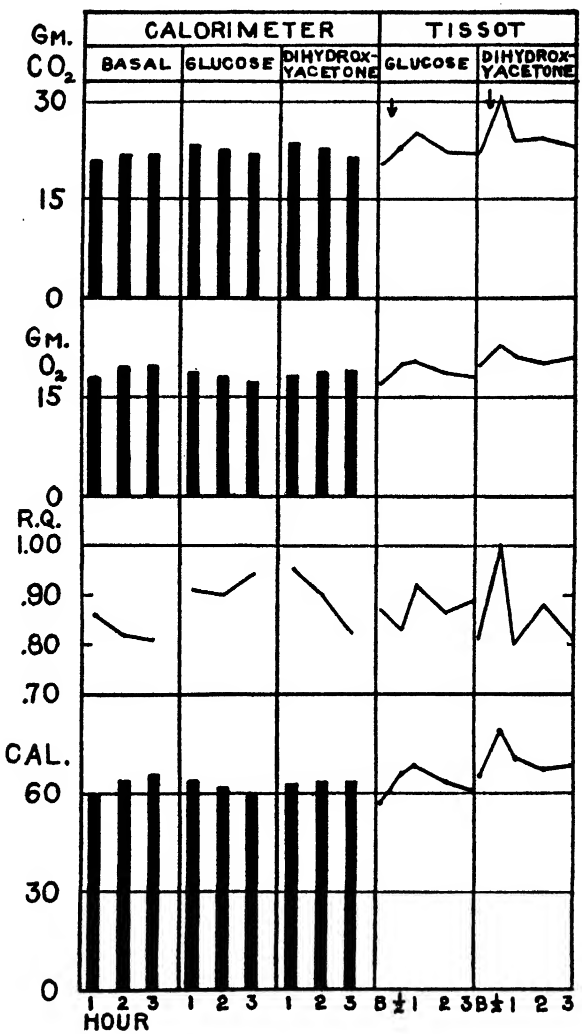

CHART III. Subject E. M., graphic representations of findings in metabolism observations.

to fat. These are the accepted explanations of what happens after the ingestion of carbohydrate when there is an elevation

in the respiratory quotient. Also an increase may occur from overventilation or from the release of carbonic acid when some

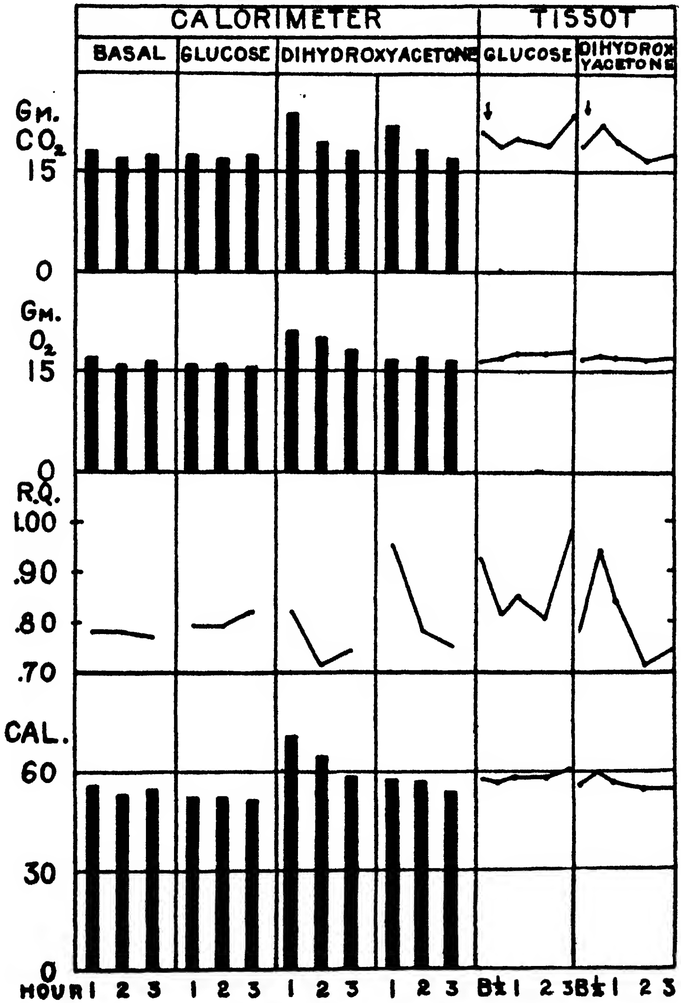

CHART IV. Subject C. W.; graphic representations of findings in metabolism observations.

other acid takes its place in maintaining the acid-base equilibrium of the body. Chart V shows the minute volume of expired air

for each period of the Tissot observations. It was found that the ventilation in normals increased 30 to 50 per cent at the end of 30 minutes after the ingestion of dihydroxyacetone. In one diabetic, C.W., the increase was 18 per cent while in the second, J.M., there was no increase noted. It is interesting that the subject J.M. was the only one who did not show an increased carbon dioxide elimination and an elevated respiratory quotient. The carbon dioxide-combining powers of the blood of three sub-

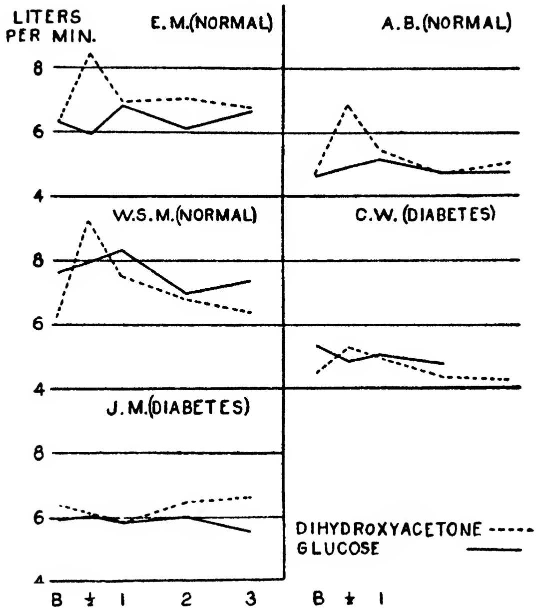

CHART V. Minute volume of expired air for each period of the Tissot observations.

jects were determined and no significant variation from normal was present. Therefore we think that overventilation is one factor in producing the elevated respiratory quotient.

There is an average increased heat production during the 1st hour following the ingestion of 50 gm. of dihydroxyacetone of 8.8 per cent. For the same amount of glucose the increase was 6.9 per cent. When calculated for periods of 3 hours, the elevation is only very slightly greater following dihydroxyacetone than

following glucose. Chart VI shows graphically the average hourly heat production for all observations calculated on the basis of 3 hour experiments. The average percentage increase above basal from 50 gm. of glucose was 3.5 per cent and from the same amount of dihydroxyacetone was 5.0 per cent. Therefore dihydroxyacetone shows almost no variation in heat production from glucose.

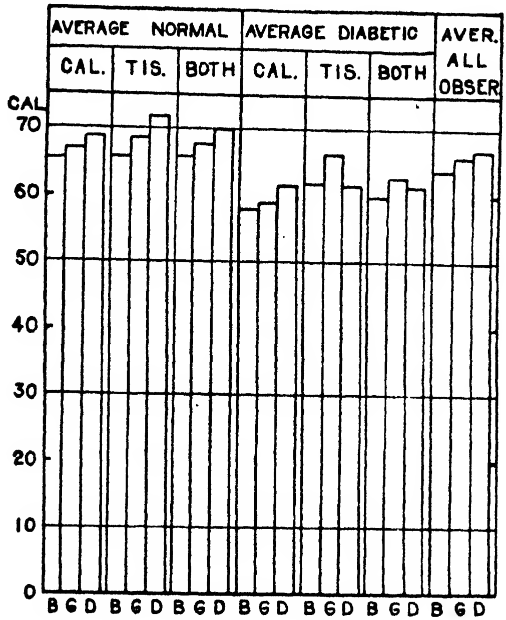

CHART VI. Average hourly heat production for all observations. *B*, basal; *G*, glucose; *D*, dihydroxyacetone.

The observation on C.W. of March 10, 1927, was made while he was in a mild ketosis. He eliminated 5.6 gm. of acetone bodies that day. A respiratory quotient of 0.82 was obtained during the 1st hour with the combustion of 5.7 gm. of carbohydrate. No basal observation was made during this period of ketosis but in other diabetic patients eliminating about the same amount of acetone bodies the basal respiratory quotients average between 0.73 and 0.75. His respiratory quotient for the 3rd

hour was 0.74. This observation suggests that even during a mild ketosis when the body is using only small amounts of carbohydrate it is able to utilize dihydroxyacetone to a greater degree than glucose.

The blood sugar curves in the three normal subjects studied showed a fall in the level at the end of 1 hour after the ingestion of 50 gm. of dihydroxyacetone. This was marked in subjects A.B. and W.S.M. If the dihydroxyacetone is being converted into glucose, this fall in blood sugar is difficult to explain unless one considers either a rapid storage or a rapid oxidation of carbohydrate due to increased production of insulin. In the two diabetics there was an elevation in the blood sugar curves which was less marked than the elevation after glucose. There was no increase in M-substances in the blood after the ingestion of 50 gm. of dihydroxyacetone but there was a slight increase of these substances in the urine in all subjects. The additional amount of M-substances eliminated in the urine was not significant in accounting for the dihydroxyacetone taken as in no subject was more than 0.1 gm. recovered which represented only 0.2 per cent of the 50 gm. ingested. No increase in any subject was found in the M-substances in blood or urine after 50 gm. of glucose were taken.

The abnormally low basal quotients obtained with the Tissot apparatus in one diabetic, J.M., require consideration. They were obtained consistently in repeated tests as may be noted in the data (Table III). There was only a trace of acetone found in the urine during the period of the studies. There was no error detected in technique, in the management of the test, or in the analyses of the gas. The observations were carried out by different observers and the analyses were done by an experienced analyst who obtained consistent checked results both with outside air and with other subjects. The patient's condition was complicated by a sinus infection which was treated during his stay in the hospital. All observations except the one on May 10, 1927, were made before this condition was treated. The observation of May 10, 1927, consisted of a continuous run without removing nose clip or mouthpiece until four separate samples were obtained at approximately 20 minute intervals. These results show as uniform results as can be expected. It was found that these quotients were from 0.03 to 0.08 points lower than those obtained

in the calorimeter. The respiratory quotient curves of this subject show an elevation of 0.05 after glucose and 0.07 after dihydroxyacetone, which are similar to those of other subjects, but the entire curve is at a much lower level. We are unable to explain these quotients.

As an additional check on the two methods of determining metabolism, the basal metabolism of the trained subject E.F.D.B. was obtained with the Tissot apparatus and the calorimeter on the same morning. The data, which are presented in Table III, show a very close uniformity in the results obtained by the two methods. We feel that there may be certain individuals who may give consistent low results as determined by short time (6 to 10 minute) periods. Therefore care should be used in selecting subjects for metabolic investigation. Any conclusions drawn as to the percentage of calories produced by the oxidation of various foodstuffs in a subject like J.M. may show considerable variation when based on data obtained by these two methods.

The way in which dihydroxyacetone is converted and used in the body has not been explained. There is more similarity between the actions of dihydroxyacetone and fructose than between dihydroxyacetone and glucose. Togel, Brezina, and Durig (22), Higgins (23), Benedict and Carpenter (24), Carpenter (25), and Deuel (26) in their studies with sugars found that fructose and sucrose produced a more rapid and greater elevation of the respiratory quotient than glucose. Cori and Cori (27) reported that in rats without insulin 51 per cent of the dihydroxyacetone absorbed is oxidized, 21 per cent deposited as liver glycogen, and 15 per cent as body glycogen. It is interesting to note that both dihydroxyacetone and fructose are ketoses while glucose is an aldehyde. It seems possible that in the body dihydroxyacetone may be converted into an easily oxidizable substance, possibly a form of glucose which has some different characteristics from the ordinary glucose. Perhaps glucose itself before it can be utilized in the body, must be converted into this readily oxidizable form. This oxidizable substance, if it were a form of glucose, could not be utilized in totally diabetic animals and when eliminated it would be detected as glucose. This would also explain the fact that it is more rapidly utilized in normals than is glucose and would account for its partial utilization in diabetics. The

data obtained in this study do not yield any information as to whether or not dihydroxyacetone is an intermediary product in carbohydrate metabolism.

SUMMARY AND CONCLUSIONS.

1. Dihydroxyacetone caused a rapid elevation of the respiratory quotient due to a marked elevation in carbon dioxide elimination. We were unable to determine the source of the excess carbon dioxide eliminated.

2. Dihydroxyacetone resulted in an increased heat production of about 5 per cent in 3 hours which was only slightly in excess of that obtained after glucose. Therefore dihydroxyacetone has approximately the same specific dynamic action as glucose when 50 gm. are taken.

3. No evidence of an increase in the M-substances (those substances, including dihydroxyacetone, which will reduce acid phosphomolybdate solutions) in the blood was obtained after the ingestion of 50 gm. of either dihydroxyacetone or glucose in either normals or diabetics.

4. There was a slight elevation in the M-substances in the urine after dihydroxyacetone. This was not present after glucose.

5. The blood sugar curves in normals showed practically no rise in level after 50 gm. of dihydroxyacetone but on the contrary they showed a fall which was quite marked at the end of 1 hour. In the diabetics there was a rise in the blood sugar level which was less marked than it was after glucose.

6. No positive information was obtained as to whether or not dihydroxyacetone is an intermediary product formed in the metabolism of glucose.

Note.—Since the preparation of this paper, W. R. Campbell reported before the Society for Clinical Investigation at Washington, D. C., April 30, 1928, that, after taking 100 gm. of dihydroxyacetone, there was a fall in the carbon dioxide-combining power of the blood and a marked elevation in blood lactic acid. This may account in part at least for the marked carbon dioxide elimination noted in our observations.

BIBLIOGRAPHY.

1. Rabinowitch, I. M., Observations on the use of dihydroxyacetone in the treatment of diabetes mellitus, *Canad. Med. Assn. J.*, 1925, xv, 374.
2. Mason, E. H., Dihydroxyacetone studies with respiratory and carbohydrate metabolism in normal men, *J. Clin. Inv.*, 1926, ii, 521.
3. Rabinowitch, I. M., Case of diabetic coma treated with dihydroxyacetone with recovery, *Canad. Med. Assn. J.*, 1925, xv, 520.
4. Kermack, W. O., Lambie, C. G., and Slater, R. H., The utilization of dihydroxyacetone by the animal body, and a method for its determination, *Biochem. J.*, 1926, xx, 486.
5. Mason, E. H., Dihydroxyacetone studies with respiratory and carbohydrate metabolism in diabetes mellitus, *J. Clin. Inv.*, 1926, ii, 533.
6. Himwich, H. E., Rose, M. I., and Maler, M. R., Changes in respiratory quotient produced by the subcutaneous injection of dihydroxyacetone and glucose, *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 38.
7. Campbell, W. R., and Markowitz, J., Preferential utilization of carbohydrates in diabetics, *J. Clin. Inv.*, 1926, ii, 608.
8. Campbell, W. R., and Markowitz, J., On the metabolism of dihydroxyacetone in pancreatic diabetes, *Am. J. Physiol.*, 1927, lxxx, 561.
9. Markowitz, J., and Campbell, W. R., The fate of dihydroxyacetone in the animal body, *Am. J. Physiol.*, 1927, lxxx, 548.
10. Campbell, W. R., and Hepburn, J., The effect of dihydroxyacetone on insulin hypoglycemia, *J. Biol. Chem.*, 1926, lxviii, 575.
11. Hewitt, J. A., and Reeves, H. G., Action of glyceric aldehyde and dihydroxyacetone on insulin hypoglycemia, *Lancet*, 1926, ii, 703.
12. Riche, J. A., and Soderstrom, G. F., Clinical calorimetry. II. The respiration calorimeter of the Russell Sage Institute of Pathology in Bellevue Hospital, *Arch. Int. Med.*, 1915, xv, 805.
13. Gephart, F. C., and Du Bois, E. F., Cinical calorimetry. III. The organization of a small metabolism ward, *Arch. Int. Med.*, 1915, xv, 829.
14. Benedict, S. R., The detection and estimation of glucose in urine, *J. Am. Med. Assn.*, 1911, lvii, 1193.
15. McCann, W. S., The effect of the ingestion of foodstuffs on the respiratory exchange in pulmonary tuberculosis, *Arch. Int. Med.*, 1921, xxviii, 847.
16. Folin, O., and Wu, H., A system of blood analysis. Supplement I. A simplified and improved method for the determination of sugar, *J. Biol. Chem.*, 1920, xli, 367.
17. Campbell, W. R., The quantitative determination of dihydroxyacetone, *J. Biol. Chem.*, 1926, lxvii, 59.
18. Van Slyke, D. D., and Cullen, G. E., Studies of acidosis. I. The bicarbonate concentration of blood plasma; its significance, and its determination as a measure of acidosis, *J. Biol. Chem.*, 1917, xxx, 289.
19. McClellan, W. S., The quantitative estimation of dihydroxyacetone in blood and urine, *J. Biol. Chem.*, 1928, lxxvi, 481.

20. Du Bois, E. F., Basal metabolism in health and disease, Philadelphia, 2nd edition, 1927, 132.
21. Lusk, G., Animal calorimetry. XXIV. Analysis of the oxidation of mixtures of carbohydrate and fat, *J. Biol. Chem.*, 1924, lix, 41.
22. Togel, O., Brezina, E., and Durig, A., Über die Kohlenhydratsparende Wirkung des Alkohols, *Biochem. Z.*, 1913, l, 296.
23. Higgins, H. L., The rapidity with which alcohol and some sugars may serve as nutriment, *Am. J. Physiol.*, 1916, xli, 258.
24. Benedict, F. G., and Carpenter, T. M., Food ingestion and energy transformation, *Carnegie Institution of Washington, Pub. No. 261*, 1918, 171.
25. Carpenter, T. M., Human metabolism with enemata of alcohol, dextrose, and levulose, *Carnegie Institution of Washington, Pub. No. 369*, 157.
26. Deuel, H. J., Jr., The respiratory metabolism following the administration of various carbohydrates, *J. Biol. Chem.*, 1927, lxxv, 367.
27. Cori, C. F., and Cori, G. T., The fate of sugar in the animal body. VIII. The influence of insulin on the utilization of glucose, fructose, and dihydroxyacetone, *J. Biol. Chem.*, 1928, lxxvi, 755.

A GENERAL REACTION OF AMINO ACIDS. II.

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(Received for publication, June 21, 1928.)

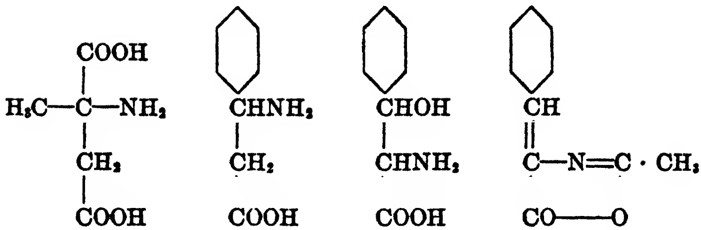
The following paper contains an account of further experiments on the reaction occurring between amino acids and a mixture of pyridine and acetic anhydride. It may be recalled that it was shown in our first paper (1) that a typical α-amino acid on being warmed with acetic anhydride and pyridine was converted into an acetylaminoacetone derivative with evolution of carbon dioxide. A β-ketonic acid was assumed to be an intermediate product.

$$R \cdot CH \cdot NH_2 \cdot COOH \rightarrow R\text{—}\underset{\substack{|\\ NH \cdot CO \cdot CH_3}}{\overset{\substack{COOH\\ |}}{C}}\text{—}CO \cdot CH_3 \rightarrow R \cdot \underset{\substack{|\\ NH \cdot CO \cdot CH_3}}{CH} \cdot CO \cdot CH_3 + CO_2$$

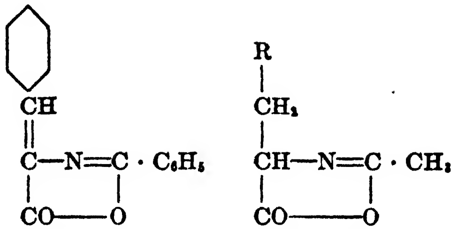
It was further shown that, if one or both of the hydrogen atoms of the amino group were substituted by alkyl groups, no typical reaction with ketone formation took place and that the same was true of an acid such as α-aminohydratropic acid which contains no unsubstituted hydrogen attached to the α-carbon atom. The bearing of these results on the mechanism of the reaction was briefly discussed. The new experiments comprise a study of the action of pyridine and acetic anhydride on a number of other types of amino acids and their derivatives. The following amino acids were found to yield substituted acetylaminoacetones which were characterized by appropriate derivatives: aspartic acid, glutamic acid, histidine, and tryptophane. On the other hand methylaspartic acid (I) gave no trace of either ketone or carbon dioxide, this result being clearly due to the absence of an unsubstituted α-hydrogen atom adjacent to the amino group. The

behavior of methylaspartic acid is analogous to that of α-aminohydratropic acid. Phenyl-β-alanine (II), chosen as a representative of the β-amino acids, gave neither ketone nor carbon dioxide but underwent simple acetylation. Serine, typical of the β-hydroxy-α-amino acids, gave unquestionable qualitative evidence of ketone formation but the amount was small as the product appeared to be unstable. Phenylserine (III), on the other hand, gave traces of carbon dioxide but no ketone. It was largely converted into the anhydride (azlactone) of acetaminocinnamic acid (IV). The fact that α,β-unsaturated azlactones of this type are not acted upon by pyridine and acetic anhydride was confirmed by experiments with the azlactone of benzoylaminocinnamic acid (V) prepared by condensing benzaldehyde with hippuric acid. The substance was entirely unacted upon and was recovered unchanged. On the other hand Bergmann, Stern, and Witte (2) have recently described some cyclic anhydrides—so called azlactones of the saturated α-amino acids. These substances are formed by the action of acetic anhydride on amino acids and are of the type shown by the general formula (VI). Azlactones derived from leucine, phenylalanine, and aspartic acid, all react with acetic anhydride and pyridine to give carbon dioxide and the same acetylaminoketones as are furnished by the amino acids themselves. It is therefore a plausible supposition that these azlactones represent an intermediate stage in the pyridine-acetic anhydride reaction. This hypothesis offers a satisfactory explanation of the curious failure of α-alkylamino acids to undergo a reaction analogous to that of the unsubstituted amino acids for the former compounds obviously cannot yield azlactones containing a hydrogen atom in the position capable of replacement by an acetyl group. On the other hand it must be admitted that as judged by the apparent evolution of carbon dioxide, the azlactones do not react noticeably more rapidly than do the amino acids themselves so that it is not improbable that they only represent one of several intermediate steps in the reaction.

A number of peculiarities are observed in the products derived from the amino acids used in the present investigation. These will be referred to in the experimental section in order to avoid repetition.



I. II. III. IV.



V. VI.

EXPERIMENTAL.

Aspartic Acid.—When finely powdered *l*-aspartic acid (3 gm.) is warmed with acetic anhydride (20 cc.) and pyridine (10 cc.) on the steam bath, the acid slowly goes into solution and at the end of about 3 hours 85 to 95 per cent of the theoretical amount (1 mol) of carbon dioxide has been evolved. After a short distillation with steam to remove the bulk of the acetic acid and pyridine, a solution is obtained giving the typical ketone reactions including a strong iodoform test. Prolonged steam distillation results in some decomposition and diacetyl will begin to appear in the distillate. The aqueous solution on being made acid to Congo red with sulfuric acid yields scarcely anything on extraction with ether, but on repeated shaking (5 to 6 times) with butyl alcohol, the product is readily extracted. When the solvent is removed a clear amber-like gum (3.1 gm.) is obtained which shows no inclination to crystallize. The product gives a strong iodoform reaction, a deep orange-red color with sodium nitroprusside in alkaline solution, turning magenta with acetic acid. Silver, mercury, and copper salts are all reduced on warming in alkaline solution, while silver nitrate produces no precipitate in acid or neutral solution.

On addition of an excess of phenylhydrazine (3 gm.) dissolved in 10 per cent acetic acid to an aqueous solution of the gum (1 gm.) no immediate precipitation takes place but on being warmed on the water bath carbon dioxide is freely evolved and a crystalline product separates out in fair quantity (0.9 gm.). Examination of this product quickly showed that it was not a simple hydrazone of an acetylaminoketone but was the *bis*-hydrazone of diacetyl. The substance melted after recrystallization from benzene at 241–243°, had all the properties of the diacetyl derivative described by Pechmann, and gave the following results on analysis.

Analysis.

$C_{16}H_{18}N_4$.	Calculated.	C 72.1, H 6.76, N 21.0.
	Found.	" 72.0, " 6.89, " 21.2.

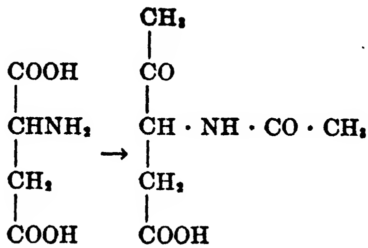
The direct formation of diacetyl itself was readily demonstrated by dissolving the original gum in dilute sulfuric acid (1:20) and distilling with steam. After a short time the distillate becomes noticeably yellow and gives all of the characteristic reactions of diacetyl.

When the gum is dissolved in dilute hydrochloric acid (10 per cent) and evaporated on the water bath a residue is obtained which contains crystals which were readily identified as ammonium chloride. The odor of diacetyl is marked during the evaporation. The non-crystalline material on being dissolved in water and extracted with ether gave a small amount of a syrupy acid agreeing in its properties with the description of β-hydroxylevulinic acid given by Wolff (3), while another portion of the syrup on being warmed on the water bath with excess of aqueous ammonia readily furnished a base, which after extraction with ether melted at 85° and gave a picrate melting at 191–192°. The base was clearly identical with tetramethylpyrazine which, as is already known, results from the action of ammonia on either diacetyl or β-hydroxylevulinic acid. The anhydrous tetramethylpyrazine gave the following results on analysis.

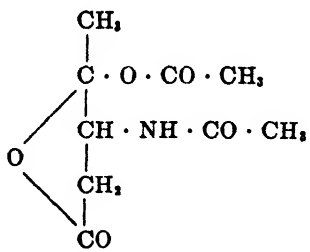
Analysis.

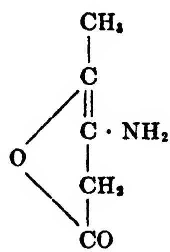
$C_8H_{12}N_2$.	Calculated.	C 70.6, H 8.83.
	Found.	" 70.2, " 8.97.

The foregoing results could well be interpreted on the basis of the idea that the original non-crystalline product of the action of acetic anhydride and pyridine on aspartic acid (VII) was an acetylaminoacetone derivative (VIII) such as should result from the general reaction observed by us in many analogous cases. The fact that the substance contains no amino nitrogen and that the ratio of total nitrogen to acetyl groups is exactly 1:1 is in harmony with this view. An inspection of the formula (VIII) shows that the ketone is a derivative of levulinic acid, namely



VII. VIII.





IX. X.

β-acetylaminolevulinic acid, and as is well known, levulinic acid and analogous γ-ketonic acids react with acetic anhydride to give substances of the type of acetyl-levulinic acid usually represented as γ-acetoxy-γ-valerolactone (4). There is reason to believe that a similar reaction takes place with the ketonic acid (VIII) under discussion and that it is converted by the further action of acetic anhydride into β-acetamino-γ-acetoxy-γ-valerolactone (IX). During the steam distillation and other operations connected with the working up of this product one of the acetyl groups is removed with regeneration of the ketone (VIII). Under certain conditions

it has been possible to convert β-acetylaminolevulinic acid (VIII) into a finely crystalline compound which may be designated as β-amino-α-angelica lactone (X), although it is possible though improbable, that it is a derivative of β-angelica lactone. The preparation of this substance presents difficulties since the use of acids or strong alkali is ruled out owing to the ease with which the nitrogen is split off as ammonia. It has not been found possible to establish conditions which regularly result in a successful preparation and many negative results have been encountered. When β-acetylaminolevulinic acid is dissolved in butyl alcohol containing a trace of sulfuric acid, and evaporated and subsequently dried for a week or so in a vacuum desiccator over sulfuric acid, a gummy residue is obtained. When this is warmed with a little 5 per cent sodium carbonate solution unchanged acid is dissolved and a crystalline residue of the lactone remains which is filtered off and recrystallized from boiling water. Its properties and analysis are in accord with the structure suggested. The substance is only moderately soluble in water, either hot or cold, and its aqueous solution reacts neutral to litmus. It is readily soluble in alcohol and less so in ether. It is instantly dissolved by strong hydrochloric acid but is in part recovered unchanged on dilution and evaporation. It is not decomposed by sodium carbonate but is easily decomposed by sodium hydroxide. It does not reduce Fehling's solution but reduces permanganate in alkaline solution somewhat slowly. The aqueous solution of the substance gives no iodoform reaction when treated with iodine and sodium hydroxide in the cold, but if it is first warmed with a little caustic alkali, so as to open the lactone ring, the cooled solution gives an intense iodoform reaction. On treatment with nitrous acid the compound gives nitrogen equivalent to 8.5 to 9.5 per cent in 5 minutes.

Analysis.

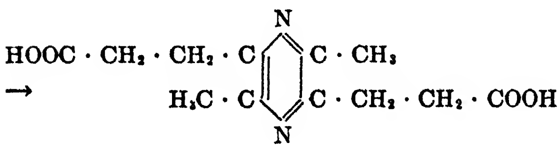
$C_5H_7O_2N$.	Calculated.	C 53.1, H 6.20, N 12.4.
	Found.	" 53.4, " 6.51, " 12.4.

Glutamic Acid (XI).—When glutamic acid (5 gm.) is warmed with acetic anhydride (25 cc.) and pyridine (15 cc.) on the steam bath, no carbon dioxide is evolved for about half an hour and even then the evolution is slow. After 3.5 hours the amount of carbon

dioxide amounts to only 15 to 20 per cent of the amount calculated for 1 molecular proportion. This marked difference shown by glutamic acid when compared with analogous amino acids is apparently due to its conversion into pyrrolidonecarboxylic acid by the dehydrating action of the acetic anhydride. Since pyrrolidonecarboxylic acid no longer contains a free amino group such as we have found to be essential for ketone formation from amino acids, it is incapable of further reaction and accounts for the bulk of the glutamic acid originally taken. An attempt to limit the formation of pyrrolidonecarboxylic acid by adding a little acetyl chloride to the anhydride was unsuccessful.

When the reaction mixture is steam-distilled, the residue is found to contain a moderate amount of a ketone mixed with much other material. The iodoform and nitroprusside reactions are strong, and phenylhydrazine acetate gives a precipitate which readily becomes oily. Alkaline solutions of silver, copper, and mercury salts are readily reduced. It was not found possible to isolate the ketone directly, but evidence of its presence and structure (XII) were obtained through its conversion into 2, 5-dimethylpyrazine-3,6-dipropionic acid (XIII) by reactions analogous to Gabriel's pyrazine synthesis from aminoacetone.

```
                CH3
                |
COOH            CO
|               |
CHNH2           CH · NH · CO · CH3
|               |
CH2       →     CH2
|               |
CH2             CH2
|               |
COOH            COOH
 XI.                  XII.
```



XIII.

The aqueous solution containing the acetaminoacetone derivative was made acid to Congo red and then extracted by repeated shaking with butyl alcohol. After removal of the solvent the syrupy residue was heated for an hour on the steam bath with 10 cc. of hydrochloric acid (1:1). The solution was then evaporated to remove most of the acid and then treated with excess of ammonia. After standing at room temperature for an hour the mixture was evaporated to small bulk (10 cc.). The solution which reacted acid to litmus was made more strongly acid with acetic acid and allowed to crystallize. The first crop of crystals consisted of practically pure silky needles of the pyrazine acid while subsequent crops were contaminated with glutamic acid and were discarded. The yield was only 2 to 3 per cent of the glutamic acid originally taken.

2,5-Dimethylpyrazine-3,6-dipropionic acid is a type of acid that has not hitherto been prepared. It is very sparingly soluble in cold water and only moderately soluble in boiling water. Its aqueous solution only just reddens blue litmus paper. It crystallizes from methyl alcohol, in which it is readily soluble on heating, in stout prisms while from water it separates in long needles. It is fairly soluble in ether. The acid melts at 211–213° without evolution of gas. Its saturated aqueous solution gives only a trifling brownish yellow color with ferric chloride and no immediate precipitate with either silver nitrate or mercuric chloride. When it was left standing, a finely crystalline silver salt separates in the form of heavy needles. The substance gives no diazo reaction and it contains no amino nitrogen (Van Slyke).

Analysis.

$C_{12}H_{16}O_4N_2$.	Calculated.	C 57.1, H 6.35, N 11.1.
	Found.	" 57.0, " 6.63, " 11.1.

Methylaspartic Acid.—This substance (I) was prepared from acetoacetic ester in the usual way. On being heated (1 gm.) with acetic anhydride (10 cc.) and pyridine (7 cc.) on the steam bath for several hours no carbon dioxide was evolved and when the products were worked up in the usual way no trace of any aminoacetone derivative was detected. This result is obviously in accordance with what would be expected.

Serine.—The synthetic acid (1 gm.) was heated with acetic

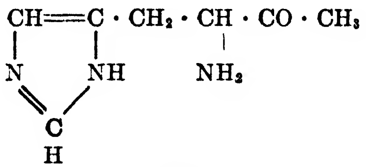
anhydride (10 cc.) and pyridine (7 cc.). After an interval of about 15 minutes carbon dioxide was freely evolved. After 4 hours the mixture was distilled with steam. The distillate contained no volatile ketone. The residue gave a strongly positive iodoform reaction but the size of the precipitate seemed small in comparison with similar tests with other amino acids. Fehling's solution was reduced on boiling while sodium nitroprusside gave a positive but rather atypical reaction. Phenylhydrazine gave small amounts of a sticky hydrazone, while boiling with *p*-nitrophenylhydrazine in 5 per cent sulfuric acid gave small amounts of an osazone giving the characteristic blue color with sodium hydroxide and alcohol. It was not found possible to prepare in crystalline form either the original product of the reaction or a satisfactory derivative. While the tests above enumerated clearly indicate that some acetaminoacetone derivative, analogous to that obtained from other amino acids, had been formed, its amount appeared small and its stability slight. Only two experiments were made with serine as the mediocre results did not seem to justify the sacrifice of larger amounts of this amino acid.

Phenylserine.—The acid (1 gm.) was warmed on the water bath with acetic anhydride (7 cc.) and pyridine (5 cc.) for 8 hours. A slight evolution of carbon dioxide took place but on steam disdillation neither distillate nor residue gave any reactions indicative of ketone formation. Crystals separated from the nonvolatile aqueous residue which melted at 152°. They were identified by properties and by analysis as the "azlactone" anhydride of acetaminocinnamic acid (IV) first described by Erlenmeyer and Früstück (5). On being dissolved in warm sodium hydroxide and precipitated with hydrochloric acid, acetaminocinnamic acid melting at 190° was obtained.

Phenyl-β-Alanine.—This amino acid (1 gm.) was treated exactly as described above in the case of phenylserine. No carbon dioxide was evolved and no ketone formation took place. The aqueous residue gave a large yield of acetyl-phenyl-β-alanine, which after crystallizing from acetic acid melted at 161–162° as described by Posner (6).

Histidine.—Experiments were made with either the free histidine base or the dihydrochloride without disclosing any difference. On being warmed (1 gm.) on the water bath with

acetic anhydride (7 cc.) and pyridine (5 cc.), carbon dioxide was evolved freely after about 10 minutes and in the course of 2 hours 93.5 to 97 per cent of the amount of carbon dioxide calculated for 1 molecular proportion was obtained. After steam distillation the residue was found to give intense iodoform and nitroprusside reactions and to reduce alkaline solutions of copper, silver, and mercury salts. Phenylhydrazine acetate gave an unattractive hydrazone slowly separating in minute needles. Semicarbazide and aminoguanidine gave no crystalline derivatives. Since the acetylaminoketone appeared to crystallize with difficulty it was decided to remove the acetyl group by evaporation with 10 per cent hydrochloric acid. The concentrated solution on standing deposited stout colorless prisms melting at 205–206° which gave results on analysis in satisfactory agreement with the expected dihydrochloride of (4)-imidazolyl-(3)-amino-butanone (2) (XIV).



XIV.

Analysis.

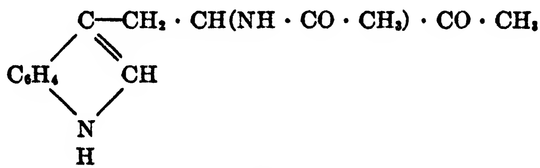
$C_7H_{11}ON_3 \cdot 2HCl$.

	C	H	N	amino N	Cl
Calculated.	37.2	5.75	18.6	6.19	31.4.
Found.	37.0	6.01	18.7	5.95	31.7.

The yield of crystalline hydrochloride amounts to about 60 per cent of the theoretical amount but much additional product remains in the mother liquor. It gives the reactions described above typical of aminoacetone derivatives and also an intense diazo reaction while on treatment with alkali yields a pyrazine derivative which will be further examined. It will be noticed that the base only differs from β-iminazolethylamine by the substitution of a hydrogen atom by an acetyl group but unlike the latter substance it was found by Dr. C. Lieb scarcely to affect the blood pressure when doses of from 1 to 20 mg. were injected intravenously into a cat.

Tryptophane.—This amino acid (1 gm.) was treated as usual with acetic anhydride (6 cc.) and pyridine (4 cc.). The reaction

was fairly vigorous and carbon dioxide was evolved freely. On steam distillation a little tar separated and was removed by filtration. On concentration of the aqueous solution, a clear yellow oil, moderately soluble in water, was obtained. It did not crystallize. Bromine water gives a thick yellow precipitate while iodine and sodium hydroxide give a deep wine-red color with only a small separation of iodoform. Fehling's solution is reduced on boiling with development of an indole-like odor. The glyoxylic reaction is quite unlike tryptophane; at first a red color develops which turns to olive-green. On addition of an excess of phenylhydrazine in 5 per cent acetic acid to the solution, a thick yellow hydrazone separates out which is sparingly soluble in water but very soluble in organic solvents, with the exception of petroleum ether. It may be crystallized from a little benzene by addition of petroleum ether. The hydrazone on analysis gave results corresponding fairly closely with those calculated for the hydrazone of an acetyl derivative of the anticipated ketone (XV).



XV.

Analysis.

$C_{22}H_{24}O_2N_4$.	Calculated.	C 70.2, H 6.39, N 14.9.
	Found.	" 69.9, " 6.65, " 14.5.

The experiments with tryptophane must only be regarded as of a preliminary character.

Azlactones.—The azlactones of leucine, phenylalanine, and aspartic acid were prepared by the method given by Bergmann, Stern, and Witte (2). They behaved with pyridine and acetic anhydride precisely like the amino acids from which they were prepared and furnished the same acetylaminoacetone derivatives, the characterization of which has already been described. The details of these experiments therefore need no minute description. On the other hand the azlactone of benzoylaminocinnamic acid gave neither carbon dioxide nor ketone as was to be anticipated.

BIBLIOGRAPHY.

1. Dakin, H. D., and West, R., *J. Biol. Chem.*, 1928, lxxviii, 91.
2. Bergmann, M., Stern, F., and Witte, C., *Ann. Chem.*, 1926, cdxlix, 277.
3. Wolff, L., *Ann. Chem.*, 1891, cclxiv, 235.
4. Bredt, A., *Ann. Chem.*, 1890, cclvi, 314.
5. Erlenmeyer, E., and Früstück, E., *Ann. Chem.*, 1895, cclxxxiv, 36.
6. Posner, T., *Ber. chem. Ges.*, 1905, xxxviii, 2316.

SOME AROMATIC DERIVATIVES OF SUBSTITUTED ACETYLAMINOACETONES.

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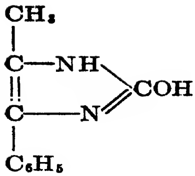
(Received for publication, June 22, 1928.)

In preceding papers (1) we have described a reaction by which α-amino acids may be converted into acetylaminoacetone derivatives by the action of acetic anhydride and pyridine. The researches of Gabriel and his coworkers and others furnish abundant evidence of the great reactivity of aminoacetone and its homologues and their value in synthetic work. Since a number of these ketone derivatives are now easily prepared from readily accessible amino acids it appeared worth while to examine some of their transformations in order to illustrate their possibilities for preparative purposes. The present communication simply deals with some of the substances prepared from aromatic α-amino acids such as phenyl-α-aminoacetic acid, phenylalanine, and tyrosine. The amino acids were first converted into their corresponding acetylaminoacetone derivatives by the action of acetic anhydride and pyridine as already described in our first paper. The further transformations of the acetylaminoacetone compounds follow well established reactions and offer nothing essentially new as regards methods.

$$
\begin{array}{lll}
CH_3 & CH_3 & CH_3 \\
| & | & | \\
CO & C{=}NOH & CO \\
| & | & | \\
CH \cdot NH \cdot CO \cdot CH_3 & CH \cdot NH \cdot CO \cdot CH_3 & CHNH_2 \\
| & | & | \\
C_6H_5 & C_6H_5 & C_6H_5 \\
\text{I.} & \text{II.} & \text{III.}
\end{array}
$$



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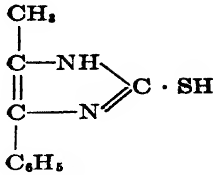

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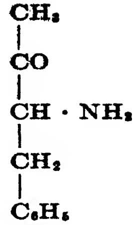
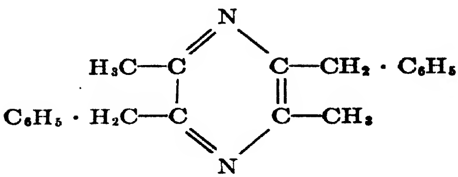
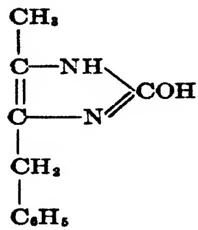


VI.



VII.



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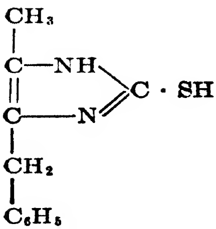

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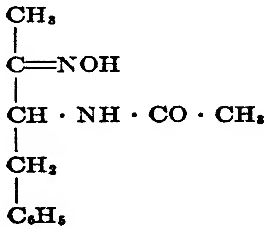

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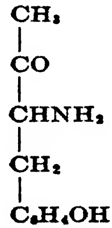

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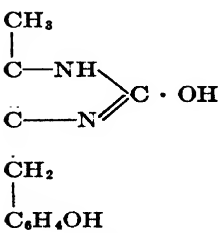

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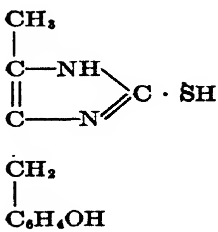

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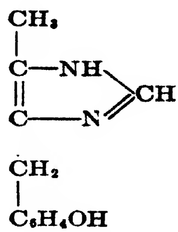

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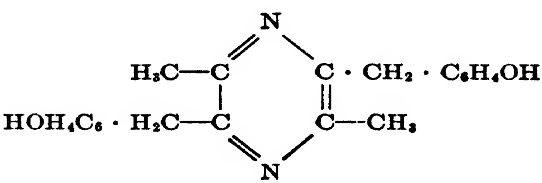

XV.

EXPERIMENTAL.

α-Phenyl-α-Acetaminoacetone (*I*).—Details of the preparation of this substance from phenylaminoacetic acid have already been given (1) together with its conversion by hydrolysis into the hydrochloride of α-phenyl-α-aminoacetone (III) already described by Kolb (2).

α-Phenyl-α-Acetaminoacetoxime (*II*).—The ketone (I) (1 gm.) was dissolved in warm alcohol (3 cc.) and treated with hydroxylamine hydrochloride (1 gm.) dissolved in a little water together with sodium bicarbonate (0.8 gm.). The oxime quickly crystallizes out in the form of nacreous crystals, very sparingly soluble in water and moderately soluble in alcohol. It melts at 152°. Yield 0.8 gm.

Analysis.

$C_{11}H_{14}O_2N_2$.	Calculated.	C 64.1, H 6.80.
	Found.	" 64.3, " 6.93.

The oxime just described on being dissolved in alcohol and reduced with sodium and acetic acid gives a base with an extremely powerful depressor action which will be further investigated.

4-Phenyl-5-Methyliminazolone.—α-Phenyl-α-aminoacetone hydrochloride (III) (1 gm.) was dissolved in 5 cc. of water and warmed on the water bath with potassium cyanate (1 gm.). The iminazolone soon begins to separate out in the form of sandy rosettes of needles melting at 287–289°. Yield 0.8 gm. The substance is almost insoluble in water and in sodium carbonate solution. On treatment with diazo salts in sodium carbonate solution only a faint yellow color is produced.

Analysis.

$C_{10}H_{10}ON_2$.	Calculated.	C 68.9, H 5.75, N 16.1.
	Found.	" 68.9, " 5.90, " 16.0.

4-Phenyl-5-Methyl-2-Thioliminazole(*V*).—α-Phenyl-α-aminoacetone hydrochloride (1 gm.) was dissolved in 5 cc. of water and heated in a covered dish on the steam bath with potassium thiocyanate for a couple of hours. After a short time slightly yellow shining needles begin to separate. The yield was 0.64 gm. The substance is very sparingly soluble in hot water or alcohol but can

be recrystallized from the latter when it separates in the form of thin shining plates. It softens slightly at above 250° and melts rather indefinitely at 290–295°. It gives a moderately strong orange-yellow with alkaline diazo salts.

Analysis.

$C_{10}H_{10}N_2S$.	Calculated.	N 14.7, S 16.8.
	Found.	" 14.8, " 16.2.

This substance and the preceding one are presumably identical with the compounds obtained by Behr-Bregowski (3) from the isomeric amidoethyl-phenyl ketone hydrochloride.

α-Benzyl-α-Aminoacetone (VI).—The hydrochloride of this base was easily obtained by heating its acetyl derivative (3 gm.), prepared from phenylalanine as previously described, with 10 per cent hydrochloric acid. After being heated for an hour on the steam bath, the solution was evaporated to small bulk and then taken up in cold water. A trace of oily matter was separated by filtration and the filtrate concentrated to a thin syrup. After it has stood crystals readily separate in good yield. The substance forms hexagonal platelets extremely soluble in both water and alcohol and melts at 130°.

Analysis.

$C_{10}H_{13}ON \cdot HCl$.	Calculated.	Total N 7.03, amino N 7.03.
	Found.	" " 7.10, " " 7.09.

2,5-Dibenzyl-3,6-Dimethylpyrazine (VII).—The hydrochloride of α-benzyl-α-aminoacetone (VI) (1 gm.) was dissolved in 5 cc. of water and concentrated aqueous ammonia added (5 cc.) and the mixture allowed to stand in an open dish overnight. Oily droplets separated out which later changed to long silky needles. The crystals were dissolved in excess of hydrochloric acid, filtered from a little oily impurity, and then extracted with ether. The acid extract was disregarded and the solution again extracted with ether after being made alkaline with sodium hydroxide. The ether on evaporation left a mass of crystals which melted in the crude condition at about 85°. After being dried on a porous plate and recrystallized from a few drops of alcohol a small yield of good crystals, both plates and needles, melting at 92–94°, was obtained. The base is sparingly soluble in water and extremely soluble in

alcohol and ether and less so in petroleum ether. The base is volatile in steam and has a slight hyacinth-like odor. The vapor turns red litmus blue but it is not a strong base and requires a considerable excess of hydrochloric acid to dissolve it. The hydrochloride is very soluble and like the analogous 2,5-diphenyl-3,6-dimethylpyrazine hydrochloride, its solution in hydrochloric acid is decidedly yellow. The base gives a picrate crystallizing in needles and a thick precipitate with mercuric chloride which crystallized in needles from dilute hydrochloric acid.

Analysis.

$C_{20}H_{20}N_2$.	Calculated.	C 83.3, H 6.95, N 9.73.
	Found.	" 82.9, " 7.20, " 10.1.

It may be mentioned that on distillation of α-benzyl-α-aminoacetone hydrochloride with mercuric chloride and concentrated sodium hydroxide by the method so commonly successful with non-aromatic pyrazines only a trace of solid pyrazine was found in the distillate, most of the volatile base being ammonia.

4-Benzyl-5-Methyliminazolone (*VIII*).—The preparation of this substance from α-benzyl-α-aminoacetone hydrochloride and potassium cyanate was carried out exactly as for its lower homologue (IV). The yield was about 65 per cent. The compound is sparingly soluble in water and on heating begins to darken above 250° and turns to a black mass indefinitely around 270°. The diazo reaction is of negligible intensity.

Analysis.

$C_{11}H_{12}ON_2$.	Calculated.	C 70.2, H 6.38.
	Found.	" 70.0, " 6.51.

4-Benzyl-5-Methyl-2-Thioliminazole (*IX*).—1 gm. of α-benzyl-α-aminoacetone hydrochloride gave, on being warmed with an equal weight of potassium thiocyanate in 10 cc. of water, 0.72 gm. of the above compound which crystallized in silky opaque needles. It is very insoluble in water and melts at 279–280°, darkening a degree or two before melting. It gives a very trivial reaction with diazo salts in alkaline solution but if it is first boiled with ferric chloride to oxidize the thiol group, the solution on being made alkaline with soda gives an intense diazo reaction. This result is in accordance with Pyman's views of the Pauly reaction ac-

cording to which a replaceable hydrogen in position (2), (4), or (5) is essential for a typical positive reaction.

Analysis.

$C_{11}H_{12}N_2S$.	Calculated.	N 13.7.
	Found.	" 13.6.

α-p-Hydroxybenzyl-α-Acetaminoacetoxime (*X*).—This substance is readily obtained from *p*-hydroxybenzyl-acetaminoacetone, the preparation of which from tyrosine has already been described. When the ketone (1 gm.) is dissolved in warm water (5 cc.) and an excess of hydroxylamine hydrochloride added, separation of the oxime begins at once with liberation of free hydrochloric acid. Sodium carbonate solution was then added by degrees until the reaction to Congo red was no longer acid. The yield of oxime is almost the theoretical amount. It is sparingly soluble in water and readily soluble in alcohol. It may be crystallized conveniently from methyl alcohol when it separates in tufts of fine silky needles, resembling tyrosine, and melts at 189–190°. It is readily reduced by sodium amalgam in acid solution.

Analysis.

$C_{12}H_{16}O_2N_2$.	Calculated.	C 65.5, H 7.27.
	Found.	" 65.6, " 7.41.

α-p-Hydroxybenzyl-α-Aminoacetone Hydrochloride (*XI*).—When the acetyl derivative of this compound, prepared as already described from tyrosine (1) was hydrolyzed with 10 per cent hydrochloric acid on the steam bath, the hydrochloride of the base was obtained on evaporation. It is extremely soluble in water and in alcohol but is precipitated from its alcoholic solution by ether as a slightly yellow deliquescent syrup which did not crystallize readily. It was not analyzed. The reaction typical of aminoacetone derivatives were all positive.

4-p-Hydroxybenzyl-5-Methyliminazolone (*XII*).—The preceding hydrochloride (1 gm.) on being warmed with potassium cyanate (1 gm.) in aqueous solution (10 cc.) gave 0.4 gm. of the iminazolone derivative after being made acid to Congo red with sulfuric acid. The substance crystallizes in large stout prisms, is very sparingly soluble in cold or hot water, somewhat more soluble in alcohol, and

freely soluble in glacial acetic acid. It was recrystallized from aqueous methyl alcohol and melted at 243–244°.

Analysis.

$C_{11}H_{12}O_2N_2$.	Calculated.	C 64.7, H 5.88.
	Found.	" 64.2, " 6.01.

4-p-Hydroxybenzyl-5-Methyl-2-Thioliminazole (*XIII*).—The action of aqueous potassium thiocyanate on the aminoacetone derivative (XI) is rather slow and 2 or 3 hours heating on the steam bath is requisite. The conditions were those previously described for homologous substances. The thioliminazole separates out in crystals carrying a very faint yellowish tinge. It is very sparingly soluble in water, alcohol, or acetic acid. On recrystallization from methyl alcohol cubes and columnar prisms were obtained which darkened slightly above 260° and melted rather indefinitely at 273–275°.

Analysis.

$C_{11}H_{12}ON_2S$.	Calculated.	N 12.8, S 14.5.
	Found.	" 12.7, " 14.3.

The substance is most easily purified by being dissolved in dilute sodium hydroxide and precipitated with acetic acid. The alcoholic solution gives a blood-red color with gold chloride, turning to a dirty gray precipitate. Aqueous iodine is at first decolorized, then with excess gives a chocolate-brown precipitate turning almost black. When the substance (1.75 gm.) is boiled with ferric chloride (12 gm.) in 50 per cent alcohol solution, the sulfur is removed. The sulfur-free iminazole is obtained by removal of the iron with sodium hydroxide and acidification of the filtrate with acetic acid. It is a fine creamy white solid, very sparingly soluble in water, sodium carbonate solution, alcohol, or acetic acid. It does not melt below 290° but only darkens slightly. The yield was 0.4 gm. The substance was only analyzed for nitrogen (14.7 per cent) but undoubtedly has the structure corresponding to 4-*p*-hydroxybenzyl-5-methyliminazole (XIV).

2, 5-p-Dihydroxybenzyl-3, 6-Dimethylpyrazine (*XV*).—When the hydrochloride of (XI) is dissolved in water, a slight excess of aqueous ammonia added, and the mixture allowed to stand overnight in an open dish, a sparingly soluble precipitate separates in

about 40 per cent yield. It is easily purified by being dissolved in dilute sodium hydroxide and when carbon dioxide is passed into the solution it is precipitated as a very light powder with a faint tinge of buff color. It dissolves in concentrated sulfuric acid to give an orange-yellow solution and is soluble in warm hydrochloric acid if not too dilute. It gives no amino nitrogen on treatment with nitrous acid and apparently is the normally constituted pyrazine.

$C_{20}H_{20}O_2N_2$.	Calculated.	C 75.0, H 6.25, N 8.75.
	Found.	" 74.9, " 6.47, " 8.60.

BIBLIOGRAPHY.

1. Dakin, H. D., and West, R., *J. Biol. Chem.*, 1928, lxxviii, 91, 745.
2. Kolb, A., *Ann. Chem.*, 1896, ccxci, 276.
3. Behr-Bregowski, L., *Ber. chem. Ges.*, 1897, xxx, 1515.

STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD.

X. THE SOLUBILITY OF CARBON DIOXIDE AT 38° IN WATER, SALT SOLUTION, SERUM, AND BLOOD CELLS.

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Interest in the magnitude of the solubility of carbonic acid in biological fluids dates from the beginning of inquiries into the manner of transport of CO_2. Of late years it has been of especial importance in the calculations of the pH of bicarbonate-containing fluids by Hasselbalch's equation, $\text{pH} = \text{pK}_1' + \log \frac{[\text{BHCO}_3]}{[\text{H}_2\text{CO}_3]}$. The physically dissolved CO_2, expressed as H_2CO_3, is calculated from the CO_2 tension in the atmosphere with which the blood or solution is equilibrated by means of the solubility coefficient of the gas in the solution.[1]

[1] The "physically dissolved CO_2" taken up by water solutions is in part hydrated to H_2CO_3; consequently it is in part altered by chemical reaction with the solvent H_2O. According to Buytendyk *et al.* (1927) the greater part of the physically dissolved CO_2 at room temperature does remain as the anhydride, only a small portion changing to H_2CO_3. The convention by which the total hydrated plus anhydrous CO_2 is at the same time called "physically dissolved" and formulated as H_2CO_3 is inconsistent and inexact. The convention is, however, convenient, and causes no errors if the H_2CO_3 symbol is used consistently with the same meaning: we shall accordingly continue to use it.

Bunsen's solubility coefficient, customarily symbolized as α, expresses the cc. of a gas, measured at 0°, 760 mm., dissolved per cc. of solution when the latter is under 1 conventional atmosphere (760 mm.) of pressure. The tension of a gas being known, the volume of gas dissolved per unit volume of solution is calculated as $\alpha \times$ atmospheres of tension. In calculating pH by Hasselbalch's equation an error of 2 per cent in α_{CO_2} (*e.g.* caused by using $\alpha = 0.52$ instead of 0.51) causes an error of approximately 0.01 in the calculation of a blood serum pH value.

Determinations of the solubility of CO_2 in water and rather concentrated salt solutions at various temperatures have been made by Bohr (1891, 1899, 1905), Bohr and Bock (1891), Setschenow (1892), Geffcken (1904), and Just (1901).

In serum and blood, however, the difficulty of distinguishing between CO_2 combined with alkali (as bicarbonate) and CO_2 "physically dissolved" has deterred investigators from direct determinations of the latter. The CO_2 solubility coefficients for blood and blood serum in general use for the past 20 years have been those estimated indirectly from oxygen and hydrogen solubilities by Bohr (1905). He followed the general assumption, which he accredited to Zuntz, that different gases in a given aqueous solution, such as blood serum, will each show the same percentage of its solubility in pure water. Bohr found that oxygen was 97.5 per cent as soluble in serum as in water, and that hydrogen was 92 per cent as soluble in whole blood as in water. He assumed that CO_2, like O_2, was 97.5 per cent as soluble in serum as in water, and like H_2 was 92 per cent as soluble in whole blood as in water. On these assumptions he calculated the solubility coefficients of CO_2 at 38° to be $0.975 \times 0.555 = 0.541$ in plasma, and $0.92 \times 0.555 = 0.511$ in whole blood, 0.555 being taken as the solubility coefficient in water. Assuming one-third of the blood volume to be cells, he estimated the CO_2 solubility coefficient in them to be 0.45, or 81 per cent of the solubility in water. Fahr (1912) in a paper published from Bohr's laboratory after Bohr's untimely death, found by direct determinations on centrifuged cells that the relative solubility of H_2 in them was not 81, but 86 to 90 per cent of its solubility in water. Nevertheless Bohr's original estimation of α_{CO_2} in cells as 81 per cent of that in water has continued to find acceptance by nearly all investigators who have had occasion to use the value.

Peculiarly, the combination of errors affecting the different factors by which Bohr reached his value for α_{CO_2} in cells yielded, within the limits of probable variation in cells from different bloods, the same value for α_{CO_2} at which we have arrived in the present paper. For α_{CO_2} in serum, however, we obtain a value much lower than his.

The assumption that different gases show the same relative solubilities in different solutions has not proved exact ("relative solubility" $= \left.\frac{\text{(observed solubility)}}{\text{(solubility in water)}}\right)$. The data in Table I indicate the extent to which the relative solubilities of different gases can vary in solutions in general, and in blood in particular. If the relative solubilities of the different gases in the same solvent were equal, the values in each horizontal line of Table I would all be alike.

We have determined the solubility of CO_2 directly in acidified serum, without dilution except that caused by addition of 1 cc. of concentrated lactic acid per 100 cc. of serum. In the case of cells

TABLE I.

Relative Solubilities of Various Gases in Different Solvents.

The data, except for blood, plasma, and cells, are from the Landolt-Börnstein "Tabellen," and are for 20° temperature. The blood data are for 37–38° unless otherwise indicated.

Gas	O_2	N_2	CO	H_2	N_2O	CO_2	Acetylene.
Solvent.	$\frac{\alpha \text{ solvent}}{\alpha\ H_2O}$.	$\frac{\alpha \text{ solvent}}{\alpha\ H_2O}$.	$\frac{\alpha \text{ solvent}}{\alpha\ H_2O}$.	$\frac{\alpha \text{ solvent}}{\alpha\ H_2O}$.	$\frac{\alpha \text{ solvent}}{\alpha\ H_2O}$.	$\frac{\alpha \text{ solvent}}{\alpha\ H_2O}$.	$\frac{\alpha \text{ solvent}}{\alpha\ H_2O}$.
1 M NaCl	0.724	0.601		0.785		0.794	
1 " KCl	0.737			0.798	0.800	0.848	
1 " HCl	0.932			0.928	0.956	0.968	
0.5 " H_2SO_4	0.894			0.918	0.914	0.932	
1 " KOH	0.670			0.736	0.734		
1 " NaOH	0.662			0.721			
1 " sucrose	0.554			0.680		0.740	
Benzene		7.10	7.10	3.80		2.94	
Methyl alcohol	7.67	8.66	8.13	4.75		4.65	
Ethyl "	7.70	8.77	8.00	3.75		3.28	
Amyl "		7.50	7.13	1.51		2.22	
" acetate		9.44	8.90	3.89		4.99	
Acetone	6.91	8.94	9.26	3.85		7.62	
Ethyl ether				6.33		5.11	
Whole blood		1.27* to 1.52		0.85† to 0.91	112‡		
Plasma or serum	0.975§	0.97¶	0.80 (15°)‖ 0.72 (37°)	0.94† to 0.95	0.975‡	0.934**	0.98††
Cells				0.86† to 0.90		0.79**	0.99††

* Bohr (1897).
† Fahr (1912).
‡ Siebeck (1909).
§ Bohr (1905).
¶ Stoddard (1926–27).
‖ O'Brien and Parker (1922).
** Present paper.
†† Schoen (1923).

it was impossible to work with undiluted material because of its viscosity. We have consequently determined the solubility of CO_2 in acidified cells diluted with 3 or more volumes of water, and estimated the solubility in undiluted cells by extrapolation.

We have also studied the effects of salts and acids in water solution on the solubility of CO_2 in order to estimate the effect of the electrolytes on the solubility of the gas in blood.

EXPERIMENTAL TECHNIQUE.

Preparation of Material.

For the determination of the solubility of CO_2 in *water* ordinary distilled water was acidified with HCl to 0.01 N concentration in order to overcome the trace of alkali usually present. The concentration of HCl used is sufficient to affect the solubility coefficient by only 1 part in 2000.

The solutions of acids were standardized by titration with phenolphthalein as indicator. The lactic acid solutions were boiled in order to break up the anhydride.

Of the *salt solutions* reported in Table IV, the NaCl and KCl solutions were prepared by dissolving weighed portions of the salts in 0.01 N hydrochloric acid. The lactate solutions were made by adding known excesses of lactic acid to solutions of $KHCO_3$ and $NaHCO_3$. The KH_2PO_4 solution was prepared by dissolving weighed amounts of the salt in standard dilute H_3PO_4. The NaH_2PO_4 solutions were prepared by addition of NaOH to standard H_3PO_4. The acid oxalate solutions were prepared from $K_2C_2O_4$ plus equimolar weights of free oxalic acid crystals. In all the salt solutions the pH was determined and found to be below 3.5.

Serum and cells were obtained by centrifugation of defibrinated blood. The *plasma* reported in Table VII was obtained from whole blood which had been treated with approximately 0.15 per cent of potassium oxalate, equivalent to about 0.2 gm. per 100 cc. of plasma present. The two plasmas reported in Table VII received about 0.5 per cent of potassium oxalate.

Saturation with CO_2.

Two procedures were used for saturating solutions with CO_2 gas. One consisted of bubbling it through the solution, as shown

in Fig. 1. In the other procedure, which we shall call the "tonometer saturation," a relatively small volume of solution was rotated in a large vessel filled with the gas at atmospheric pressure. Both procedures gave identical results with solutions of acids and salts. Only the tonometer saturation was used with plasma and

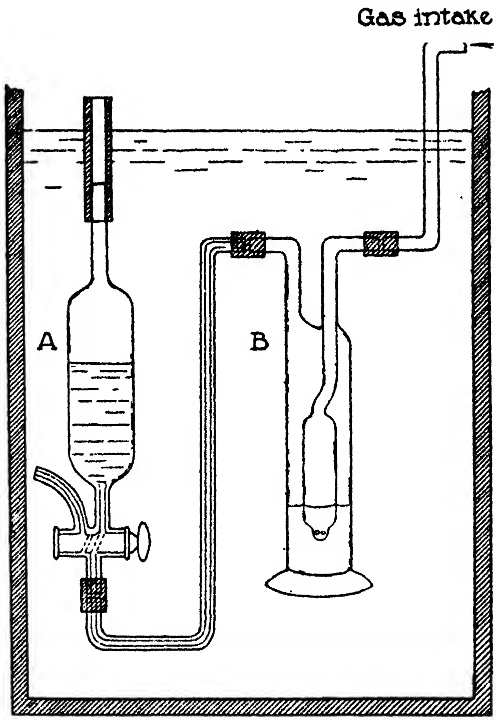

Fig. 1. Apparatus used for saturation with gas by bubbling method.

with cell solutions, saturation by bubbling being difficult because of frothing.

Saturation by Bubbling.—The solution, usually 25 to 50 cc., was placed in the saturating tube shown in Fig. 1, the lower end of which was connected through a wash bottle with a Kipp CO_2 generator. The outlet from the upper end was sufficiently wide (6 mm. bore) to prevent back pressure.

The tube and wash bottle were immersed in a constant tempera-

ture water bath[2] maintained at 38.0°±0.1°. After the solution had reached the temperature of the bath, CO_2 was bubbled through it rapidly for 30 minutes, which was twice the time found sufficient to complete the saturation. At the end of this time the cock at the bottom of the tube was closed and the wash bottle, *B*, was replaced by a mercury reservoir. Without removing the saturating tube from the bath, the free gas was driven completely out of it by admitting mercury underneath the solution, until the latter was driven up into the rubber outlet tube. The rubber tube was then closed with a clamp, and the solution was ready for determination of its CO_2 content.

The CO_2 from the Kipp generator was tested by absorbing 1 liter with alkali. The unabsorbed residue was always less than 1 part in 2000 of the CO_2.

Saturation in Rotating Tonometer.—The technique used was that described by Austin *et al.* (1922) as their "First Saturation Method," with a double-chambered tonometer to permit separation of the liquid and gas phases after saturation was finished The gas phase was renewed once during the saturation

Analyses.

The *CO_2 contents* of the solutions were determined in the manometric gas apparatus of Van Slyke and Neill (1924). The special 5 cc. pipette and technique for transfer of the solution from the saturating tube to the manometric gas apparatus, described in section XII of Van Slyke and Neill's paper were employed. The analyses were carried out in a Van Slyke-Neill gas apparatus with an extraction chamber of 100 cc. capacity instead of the usual 50 cc. The CO_2 gas extracted from each 5 cc. sample of saturated solution was brought to 4 cc. volume for measurement of its pressure, which was determined by the difference between the manometer readings before and after absorption of the CO_2 with NaOH solution. The pressures measured were in the neighborhood of 500 mm. when the solutions had been saturated with pure CO_2 at 38°. Duplicate analyses at the same tem-

[2] The thermometers in the bath were calibrated by comparison with two Bureau of Standards thermometers. The temperature was found not to vary in any part of the bath by more than 0.1° from 38°.

perature usually agreed within 1 mm. of pressure. The CO_2 contents of the solutions were calculated from the pressures by means of the factors recently published by Van Slyke and Sendroy (1927).

Of the solutions equilibrated with CO_2 under tensions of only 40 to 50 mm. (Table XII), 15 cc. samples were taken for analysis. The extracted CO_2 was brought for measurement to a volume of 1.000 cc. and gave pressures in the neighborhood of 350 mm.

All CO_2 determinations were performed in duplicate or triplicate. Except in Table XII, we give only the mean result of the analyses of each solution. With this exception, whenever two analyses are reported for the same solution, they indicate that separate portions were saturated independently with CO_2.

The *water contents* of sera, plasmas, and cell solutions were determined by drying samples of 2.000 cc. volume overnight at 110° and determining the loss of weight. The water contents of the salt and acid solutions were estimated from the specific gravity; from the total weight of a cc. of solution the known weight of salt or acid in it was subtracted to obtain the weight of water.

Chlorides were determined by the wet ashing method of Van Slyke (1923–24).

The *protein* contents of the plasmas and sera were estimated by multiplying with 6.25 the total nitrogen determined by the Kjeldahl method. The protein content of cells was estimated as N $\times$ 5.78.

The *fat* was determined gravimetrically on 10 cc. samples by the Röse-Gottlieb method.[3]

The *total base* in serum and plasma was determined by the gasometric method of Van Slyke, Hiller, and Berthelsen (1927).

Sodium and potassium in cells were determined by ashing 5 cc. samples with nitric acid, converting the ash into sulfates, removing CO_2, Ca, and PO_4 with ammonium carbonate and barium hydroxide. The Na and K sulfates were weighed, and the K determined as chloroplatinate, as outlined in the "Methods of Analysis of the Association of Official Agricultural Chemists."

[3] The Röse-Gottlieb method was used as described on p. 262 of "Official and tentative methods of analysis of the Association of Official Agricultural Chemists," Washington, 1924.

Calculations.

The CO_2 tension in solutions saturated with pure CO_2 at atmospheric pressure was calculated as $B - W$, where B is the barometric pressure and W the vapor tension of water at the temperature of saturation (at 38° W is 49.7 mm.). In solutions saturated with a mixture of CO_2 and H_2 the CO_2 content of the dry gas mixture was determined by Haldane analysis as modified by Y. Henderson (1918), and the CO_2 tension, p, was calculated by the usual formula

TABLE II.

Solubility of CO_2 at 38° in Water Acidified by Addition of HCl to 0.01 N Concentration.

H_2O content of solution = 0.997 gm. per liter at 24°.

Method of saturation.	CO_2 content.*	CO_2 tension.	α	α^0
	vol. per cent	*mm.*	*cc. CO_2 per cc. solution*	*cc. CO_2 per gm. H_2O*
T	51.53	715.1	0.5452	0.5468
B	50.48	700.8	0.5450	0.5466
T	51.23	712.0	0.5444	0.5460
B	52.02	721.8	0.5453	0.5469
T	52.10	723.0	0.5453	0.5469
B	51.90	719.3	0.5459	0.5475
Average			0.5452	0.5468
Average estimated for pure H_2O by correcting for depression of 0.0003 in α due to 0.01 mol of HCl present			0.5455	0.5471

* The CO_2 contents given are as determined in solutions cooled to room temperature of 22–24°. The volume shrinkage is corrected for in calculating α by Equation 2.

$$(1) \qquad p = \frac{\text{per cent } CO_2 \text{ in gas}}{100} \times (B - W)$$

Bunsen's solubility coefficient, α, was calculated by the formula

$$(2) \qquad \alpha = \frac{760}{p} \times \frac{[CO_2]}{100} \times \frac{V_R}{V_{38^\circ}}$$

TABLE III.

Solubilities of CO_2 at 38° in Solutions of Hydrochloric, Lactic, and Phosphoric Acids.

Method of saturation.	Acid.	Concentration at 22°.	H_2O content at 22°.*	CO_2 content.	CO_2 tension.	α	α^0	$\Delta\alpha$ Observed.	$\Delta\alpha$ Per mol acid present per liter.	$\Delta\alpha^0$ Observed.	$\Delta\alpha^0$ Per mol acid present per kg. H_2O.
		mM per l.	*gm. per cc.*	*vol. per cent*	*mm.*	*cc. CO_2 per cc. solution*	*cc. CO_2 per gm. H_2O*	*cc. CO_2 per cc. solution*	*cc. CO_2 per cc. solution*	*cc. CO_2 per gm. H_2O*	*cc. CO_2 per gm. H_2O*
B	HCl	256	0.993	51.36	721.8	0.5384	0.5427	0.0071	0.028	0.0044	0.0017
"	"	300	0.992	51.31	723.7	0.5364	0.5407	0.0091	0.030	0.0064	0.0021
Average..........									0.029		0.0019
B	Lactic.	100	0.994	52.05	716	0.550	0.553				
T	"	100	0.994	52.18	717	0.549	0.552				
"	"	100	0.994	51.55	716	0.545	0.548				
"	"	100	0.994	51.83	718	0.546	0.549				
"	"	100	0.994	51.21	709	0.5465	0.550				
Average for 100 mM..........						0.547	0.550	−0.0015	−0.015	−0.0029	−0.0029
B	Lactic.	150	0.987	51.22	712.9	0.5436	0.5508	±0.000	±0.000	−0.0053	−0.0035
"	"	150	0.987	51.68	714.4	0.5473	0.5540				
"	"	300	0.976	51.20	712.9	0.5434	0.5569	+0.0021	+0.007	−0.0098	−0.0033
Average..........									−0.003		−0.0032
B	H_3PO_4	150	0.990	51.61	723.7	0.5396	0.5451	0.0059	0.0039	0.0020	0.013
"	"	300	0.983	50.86	723.7	0.5317	0.5410	0.0138	0.0046	0.0061	0.020
Average..........									0.0044		0.017

* H_2O contents were calculated from specific gravity minus the content of acid in gm. per cc. $\Delta\alpha$ and $\Delta\alpha^0$ indicate depressions of solubility below those in water.

TABLE IV—*Solubility of CO_2 in Salt Solutions at 38°.*

Method of saturation.	Salt. Kind.	Salt. Concentration. *mols. per l.*	Salt. Concentration. *mols. per kg. H_2O*	Free acid. Kind.	Free acid. Concentration. *mols. per l.*	Free acid. Concentration. *mols. per kg. H_2O*	H_2O content at 22–24°. *gm. per cc.*	CO_2 content at 22–24°. *vol. per cent*	CO_2 tension at 38°. *mm.*	α of acidified salt solution. *cc. CO_2 per cc. solution*	Δα Observed total, 0.5455−α. (a) *cc. CO_2 per cc. solution*	Δα Due to free acid. (b) *cc. CO_2 per cc. solution*	Δα Due to salt. (a) − (b) *cc. CO_2 per cc. solution*	Δα Per mol salt. (a) − (b) *cc. CO_2 per cc. solution*	α° *cc. CO_2 per gm. H_2O*	Δα° Observed total, 0.5471−α°. (c) *cc. CO_2 per gm. H_2O*	Δα° Due to free acid. (d) *cc. CO_2 per gm. H_2O*	Δα° Due to salt. (c) − (d) *cc. CO_2 per gm. H_2O*	Δα° Per mol salt. (c) − (d) *cc. CO_2 per gm. H_2O*
B	NaCl	0.150	0 1508	HCl	0.010	0.010	0.995	50.56	721.8	0.5300	0.0155	0.0003	0.0152	0.101	0.5327	0.0144	0.0002	0 0142	0.094
T	"	0.150	0.1508	"	0.010	0.010	0.995	49.85	715.1	0.5276	0.0179	0.0003	0.0176	0.116	0.5302	0.0169	0.0002	0.0167	0.110
B	"	0.300	0.3024	"	0.010	0.010	0.992	48.80	721.8	0.5116	0.0339	0.0003	0 0336	0.112	0.5157	0.0314	0.0002	0.0312	0.103
T	"	0.300	0.3024	"	0.010	0.010	0.992	48.33	715.1	0.5113	0.0342	0.0003	0.0339	0.113	0.5154	0.0317	0.0002	0.0315	0.104
													Mean.	0.113					0.103
B	KCl	0.150	0.1511	HCl	0.010	0.010	0.993	49.32	700.8	0.5325	0.0130	0 0003	0.0127	0.085	0.5363	0.0108	0.0002	0.0106	0.070
T	"	0.150	0.1511	"	0.010	0.010	0.993	49.80	709.0	0.5314	0.0141	0.0003	0.0138	0.092	0.5343	0.0128	0.0002	0.0126	0.083
B	"	0.300	0.3031	"	0.010	0.010	0.990	48.22	700.8	0.5207	0.0248	0.0003	0.0245	0.082	0.5260	0.0211	0.0002	0.0209	0.069
T	"	0.300	0.3031	"	0.010	0.010	0.990	48.61	709.0	0.5187	0.0268	0.0003	0.0265	0.088	0.5241	0.0230	0.0002	0.0228	0.075
													Mean.	0.087					0.074
B	Na lactate.	0.150	0.1526	Lactic.	0.100	0.102	0.983	48.30	700.8	0.5214	0 0241	0.000	0.0241	0.161	0.5303	0.0168	−0.0033	0.0201	0.132
T	" "	0.150	0.1537	"	0.150	0.154	0.976	48.78	707.0	0.5220	0.0235	0.000	0.0235	0 157	0.5350	0.0121	−0.0049	0 0170	0.111
B	" "	0.300	0.3090	"	0.100	0.103	0.971	46.53	700.8	0.5023	0.0432	0.000	0 0432	0.144	0.5174	0 0297	−0.0033	0.0330	0.107
T	" "	0.300	0.3137	"	0.300	0 314	0 956	46.53	707.0	0.4980	0.0475	0.000	0.0475	0.159	0.5207	0.0264	−0.0098	0.0362	0.116
													Mean.	0.155					0.116
B	K lactate.	0.150	0.1534	Lactic.	0.100	0.102	0.978	50.35	721.8	0.5278	0 0177	0.000	0.0117	0.118	0.5398	0.0073	−0.0033	0.0106	0.069
T	" "	0.150	0.1541	"	0.150	0.154	0.974	50.35	720.4	0.5288	0.0167	0.000	0.0167	0.111	0.5429	0 0042	−0.0049	0.0091	0.059
B	" "	0.300	0 3119	"	0.100	0 104	0.962	48.71	721.8	0.5106	0 0349	0.000	0.0349	0 116	0.5308	0.0163	−0.0033	0.0196	0.063
T	" "	0.300	0.5152	"	0.300	0.315	0.952	48.59	720.4	0.5103	0.0352	0.000	0 0352	0.117	0.5362	0.0109	−0.0098	0.0207	0.066
													Mean.	0.116					0.064
B	NaH_2PO_4	0 0375	0.0376	H_3PO_4	0.011	0.011	0.996	50.33	710.7	0.5360	0.0095	0.0005	0.0090	0.243	0.5381	0.0090	0.0002	0.0088	0.234
"	"	0.075	0.0755	"	0.019	0.019	0.994	49.50	710.7	0.5272	0 0183	0 0008	0.0175	0.233	0.5302	0.0169	0.0003	0.0166	0.220
"	"	0 120	0.1210	"	0.024	0.024	0.992	48.61	710.7	0.5175	0.0260	0.0010	0 0270	0 225	0.5217	0 0254	0.0004	0 0250	0.207
"	"	0 150	0.1512	"	0.015	0.015	0.992	48.12	712.6	0.5109	0.0346	0 0006	0.0340	0.226	0.5151	0 0320	0.0002	0 0318	0.210
"	"	0.300	0.3042	"	0.030	0.030	0.986	45.49	718.3	0.4791	0.0664	0.0013	0 0651	0 217	0.4864	0 0607	0.0005	0 0602	0.198
"	"	0.300	0.3042	"	0.030	0.030	0.986	45.42	712.6	0.4823	0.0632	0 0013	0.0619	0 206	0.4891	0 0580	0.0005	0 0575	0.189
										Mean of 0.15 and 0.30 M solutions.				0.218					0 201
B	KH_2PO_4	0.150	0.1516	H_3PO_4	0.015	0.015	0.990	49.16	720.3	0.5163	0.0292	0.0006	0.0286	0 291	0.5237	0.0234	0.0002	0.0232	0.153
"	"	0.300	0.3055	"	0.030	0.030	0.982	46.70	720.3	0.4906	0.0549	0.0013	0.0536	0.179	0.4996	0.0475	0.0005	0 0470	0.154
"	"	0.300	0.3055	"	0.030	0.030	0.982	46.30	712.6	0.4915	0.0540	0.0013	0.0527	0.179	0.5005	0.0466	0.0005	0 0461	0.154
													Mean.	0.185					0.154
B	KHC_2O_4	0.300	0.3057	None.	0	0	0.979	49.49	718.5	0.5211	0.0244	0.000	0.0244	0.081	0.5328	0.0143	0.000	0.0143	0.048
"	"	0.600	0.6198	"	0	0	0.968	47.19	718.5	0.4969	0.0486	0.000	0.0486	0.081	0.5134	0.0337	0 000	0.0337	0.054
													Mean.	0.081					0.050

Δα = depression of CO_2 solubility caused by salts or acids in solution.

where p represents the CO_2 pressure in mm. of mercury at the time of saturation, and $[CO_2]$ the volume per cent of CO_2 found in the solution analyzed at room temperature. V_R and $V_{38°}$ represent respectively the volumes occupied by 1 gm. of the solution at room temperature (when analyzed) and at the temperature of saturation. If a solution is cooled from a saturation temperature of 38° to a room temperature of 20° before it is

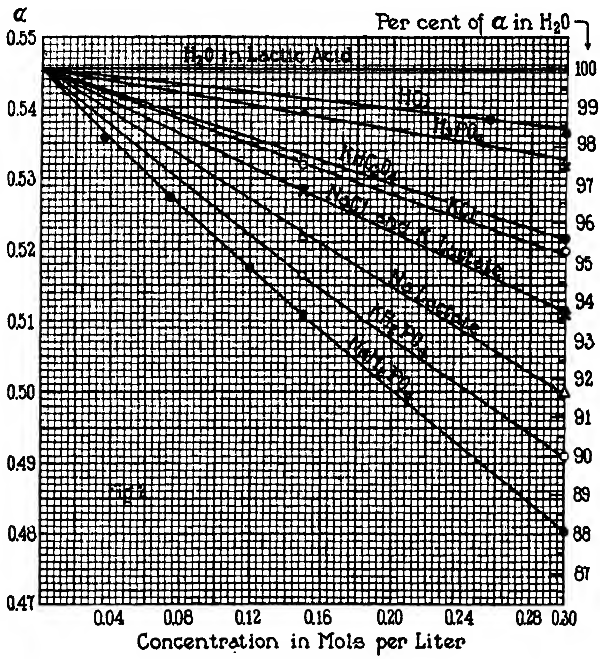

FIG. 2. Solubility of CO_2 at 38° in solutions of salts and acids. Points represent average results of analyses listed in Tables III and IV.

analyzed, it contracts by 0.5 per cent of its volume, and the volume per cent CO_2 content found by the analysis becomes 0.5 per cent higher than it was at 38°. Hence the necessity for including the factor $\frac{V_R}{V_{38°}}$ in the usual formula for calculating α. In calculating the value of $\frac{V_R}{V_{38°}}$ the coefficient of expansion of the solutions

analyzed has been assumed to be the same as that of water. At 20° the value of $\frac{V_R}{V_{38°}}$ is 0.995.

For convenience in indicating the effects of dissolved substances on the solubility of CO_2 per unit of water in the solutions, we use the symbol α^0 to indicate the cc. of CO_2, reduced to standard 0° and 760 mm., that are dissolved, under 760 mm. of CO_2 tension,

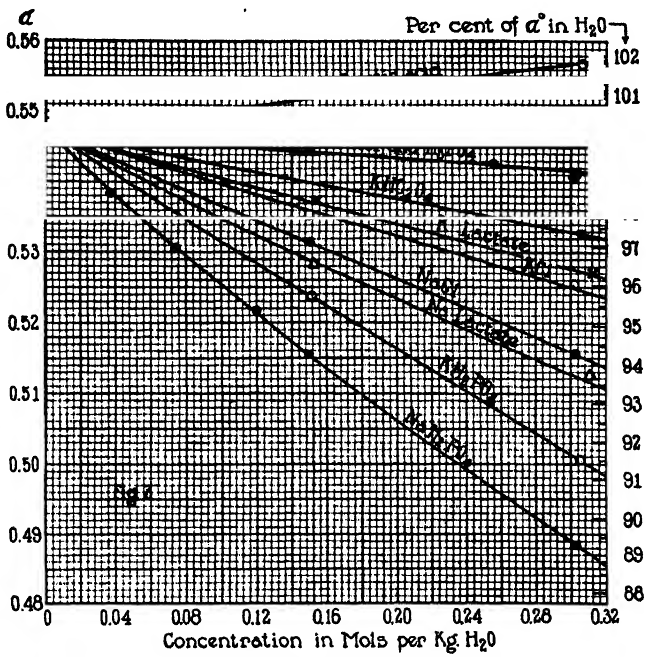

FIG. 3. Solubility of CO_2 at 38° per gram of water in solutions of salts and acids. Points represent average results of analyses listed in Tables III and IV.

per gram of water in the solution. α^0 is therefore a CO_2:H_2O ratio, and is calculated as

$$\alpha^0 = \frac{760}{p} \times \frac{[CO_2]}{100\ (H_2O)} \tag{3}$$

(H_2O) represents the grams of water per cc. of solution, the sample of solution for (H_2O) determination being measured at the same

room temperature as the sample for CO_2. Because both analyses are made thus at the same temperature, and α^0 expresses the ratio $CO_2 : H_2O$ rather than CO_2 : volume, the $\frac{V_R}{V_{38°}}$ factor does not enter into the calculation of α^0.

DETAILS AND RESULTS OF SOLUBILITY DETERMINATIONS.

Solubility of CO_2 in Water and in Solutions of Acids and Salts.—Our data on these values are given in Tables II to IV.

In Figs. 2 and 3 the solubility values are represented by curves which permit comparison of the effects of the different salts and acids on the solubility of CO_2 in aqueous solutions. The particular salts and acids studied were chosen because it was necessary to know their effects on the solubility of CO_2 in order to estimate the effects of the other constituents of cells and serum.

Water.—For the solubility coefficient of CO_2 in water at 38° our value 0.5455 is 1.7 per cent lower than the value 0.555 published in 1905 by Bohr. Bohr's value, however, was not actually determined at 38°. It was obtained by interpolation on a curve constructed from a number of α values measured by Bohr (1899) at temperatures from 0.1–61.4°. To judge from the grouping of points about the curve, the difference between Bohr's results and ours may not be outside the experimental error of his method plus the error involved in the interpolation. We have repeated six times in duplicate the determination at 38° ± 0.1°, and all six values, given in Table II, are between 0.5444 and 0.5459. Half of our saturations were by the bubbling technique and half by the tonometer technique. For the temperature 38° it appears probable that our average value 0.5455 is exact within 1 unit in the third decimal place.

Salt Solutions.—The curves of Fig. 2 indicate the presence of additive ionic effects in lowering the solubility of CO_2 in water solutions. The sodium salts regularly depress α more than do the corresponding potassium salts, and among the anions chloride, lactate, and acid phosphate rank in ascending order in their ability to depress α. Geffcken (1904, p. 285), from his solubility figures in more concentrated salt solutions, pointed out the occurrence of such ionic series, and the possibility of additive ionic effects. Our results indicate that, for the salts we have used, in the con-

centration range below 0.3 M, the effects on the solubility of CO_2 can be calculated practically within the limit of experimental error by adding the effects of the component ions.

In order to calculate the effects of the different cations and anions on α_{CO_2} we have to make an arbitrary assumption of the magnitude of the effect of some particular ion, and use this assumed value as a starting point for estimating the relative effects of the other ions. If we assume a value for some cation, and the value is higher than the actual one, the values for all the other cations will be too high by the same margin, and the values for all the anions will be correspondingly low; but the value for any given electrolyte obtained by adding those of its ions will be correct because the positive error of the additive figure for one ion will be balanced by the negative error of the figure for the opposite ion.

We have accordingly assumed that the effect of the hydrogen ion on the solubility of CO_2 in water is zero. It appears that, in fact, its effect is relatively slight. The data in the literature (Geffcken, 1904) on the solubility of CO_2 in solutions of strong acids and their salts show that of all the inorganic cations the hydrogen ion has the smallest effect. Thus, in Fig. 2, it is obvious that HCl has much less effect in depressing α_{CO_2} than has KCl or NaCl. The effect of a 0.1 N solution of HCl is in fact hardly outside the limit of experimental error. We have accordingly assumed that the H^+ ion is without effect, and that the depression of α_{CO_2} caused by HCl is due entirely to the Cl^- ion. The "molar α depression" given in Table III for HCl was subtracted from the molar depressions of NaCl and KCl (Table IV) respectively in order to estimate the depressions of α_{CO_2} caused by Na^+ and K^+ ions per unit of molar concentration. The values for the lactate$^-$, $H_2PO_4^-$, and $HC_2O_4^-$ anions were estimated by subtraction of the Na^+ and K^+ values from the molar depressions of the respective alkali salts. The molar depressions for each ion obtained from its different salts showed some variation, partly due doubtless to experimental error, partly to failure of the ionic effects to be exactly additive. The mean value for each ion is given in Table V.

In Table VI the molar depressions of the different strong electrolytes experimentally found, from the data of Tables III and IV, are compared with the molar depressions estimated by

adding the ionic depressions from Table V. The maximum deviation between calculated and observed molar depressions of α_{CO_2} is 0.006. This corresponds for a 0.3 M salt solution, the

TABLE V.

Effects of Different Ions in Depressing Solubility of CO_2 in Water at 38°.

Figures are valid for concentrations below 0.3 M. Ions are arranged in the order of their effects on α^0.

Ion.	Depression of CO_2 solubility per unit M concentration of ion present.		Per cent by which CO_2 solubility in pure water is depressed per unit M concentration of ion present.	
	$\Delta\alpha$	$\Delta\alpha^0$	$\Delta\alpha$	$\Delta\alpha^0$
H^+	0	0	0	0
$HC_2O_4^-$	0.026	0.000	4.7	0.0
Lactate$^-$	0.066	0.018	12.1	3.3
Cl^-	0.029	0.019	5.3	3.5
K^+	0.053	0.049	10.6	9.3
Na^+	0.085	0.090	15.6	16.7
$H_2PO_4^-$	0.137	0.110	25.1	20.1

TABLE VI.

Comparison of Observed Depressions of CO_2 Solubility by Salts in 0.15 to 0.30 M Concentration with Depressions Calculated as Sum of Ionic Effects.

Salt.	Depression of CO_2 solubility per unit M concentration of salt.				Per cent depression of CO_2 solubility per unit M concentration of salt.			
	$\Delta\alpha$		$\Delta\alpha^0$		$\frac{100\,\Delta\alpha}{0.5455}$.		$\frac{100\,\Delta\alpha^0}{0.547}$.	
	Observed.	Calculated.	Observed.	Calculated.	Observed.	Calculated.	Observed.	Calculated.
HCl	0.029	0.029	0.019	0.019	5.3	5.3	3.5	3.5
NaCl	0.111	0.114	0.103	0.109	20.7	20.9	18.8	19.9
KCl	0.087	0.082	0.074	0.068	15.9	15.0	13.5	12.4
Na lactate	0.155	0.151	0.116	0.108	28.4	27.7	21.2	19.8
K "	0.116	0.119	0.064	0.067	21.3	21.8	11.7	12.2
NaH_2PO_4	0.218	0.222	0.201	0.200	40.2	40.7	36.7	36.5
KH_2PO_4	0.185	0.190	0.153	0.159	35.9	34.8	28.0	29.1
KHC_2O_4	0.081	0.081	0.050	0.050	14.8	14.8	9.1	9.1

strongest with which we worked, to a difference in α_{CO_2} of only 0.002, which is 1 part in 270, or about the experimental error.

It appears therefore that for the solutions studied of less than

0.3 M concentration, the depression of CO_2 solubility caused by the salts studied can be calculated by addition of the effects of each

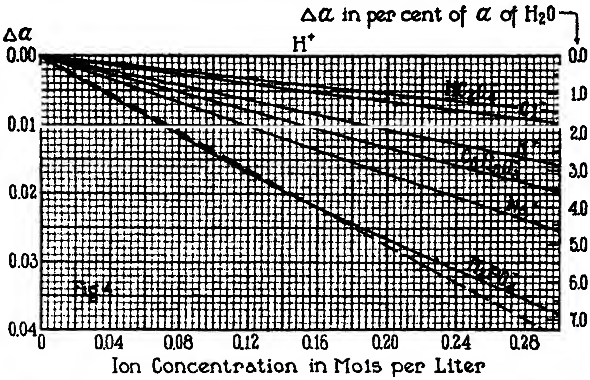

FIG. 4. Depression of Bunsen solubility coefficient by different ions. $\Delta\alpha$ = depression of α. Dash line indicates $\Delta\alpha$ for $H_2PO_4^-$ calculated from mean molar $\Delta\alpha$ when in Table V.

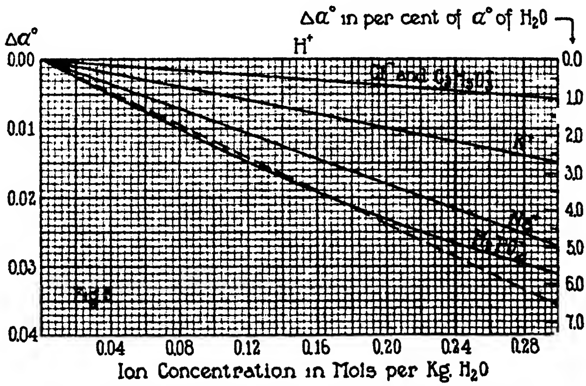

FIG. 5. Depression of CO_2 solubility per gram of water caused by different ions. $\Delta\alpha^0$ = depression of α^0. Dash line indicates $\Delta\alpha^0$ for $H_2PO_4^-$ calculated from mean molar $\Delta\alpha^0$ value in Table V.

ion, such effect being calculated by multiplying the molar depression value in Table V by the molar concentration of the ion.

The extents to which the different ions in varying concentration have been found, as above described, to depress the solubility of

CO_2 in water are shown by the curves of Figs. 4 and 5. For concentrations below 0.3 M, the effect of each ion except $H_2PO_4^-$ is a linear function of the concentration. The curves of the $H_2PO_4^-$ effects are definitely convex towards the horizontal axis. The data of Geffcken (1904) and Setschenow (1892) show a similar convexity of the curves of electrolytes in general when ranges of higher concentration are covered. Setschenow (1892) arrived empirically at the conclusion that the relative solubility, $\frac{\alpha \text{ in salt solution}}{\alpha \text{ in water}}$, varied inversely as the logarithm of the salt

TABLE VII.

Solubility of CO_2 at 38° in Normal Plasma and Serum Acidified with 0.1 Mol of Lactic Acid per Liter.

Material.	CO_2 content at 22–24°.	CO_2 tension at 38°.	H_2O content at 22–24°.	Solubilities.		Relative solubilities.		
				α	α^0	$\frac{\alpha \text{ serum}}{\alpha \text{ water}}$	$\frac{\alpha^0 \text{ serum}}{\alpha^0 \text{ water}}$	$\frac{\alpha^0 \text{ serum}}{\alpha^0 \text{ saline}}$
	vol. per cent	*mm.*	*gm. per cc.*	*cc. CO_2 per cc. solution*	*cc. CO_2 per gm. H_2O*			
Ox serum..........	47.51	706	0.931	0.509	0.549	0.933	1.004	1.031
" "	47.90	709	0.925	0.511	0.555	0.938	1.015	1.043
Normal human serum............	47.40	709	0.921	0.506	0.552	0.927	1.008	1.037
Normal human oxalate plasma......	47.32	700	0.925	0.511	0.555	0.938	1.015	1.043
Average......................			0.9255	0.509	0.553	0.934	1.011	1.038

concentration. In our relatively dilute solutions there is no evidence of such a logarithmic relationship, except in the phosphate solutions.

Normal Serum and Plasma.

Samples of normal plasma and serum were acidified by addition of 1 volume per cent of concentrated lactic acid (sp. gr. 1.20) which is approximately enough to make a 0.1 N solution. It was sufficient to depress the pH to approximately 3.5 (determined electro-

metrically). The solubility coefficient of CO_2 in the acidified serum or plasma was determined by saturation in a rotating tonometer, as described above. The results are given in Table VII.

That the changes produced in the serum by addition of the lactic acid did not significantly alter the solubility coefficient of CO_2 appears probable from the following considerations. The concentration of lactate anions produced by replacement of the bicarbonate and protein anions of serum is 35 to 45 milli-equivalents per liter, about 25 by replacement of bicarbonate and 12 to 18 by replacement of serum protein anions (Van Slyke, Wu, and McLean, 1923). From Fig. 4 we estimate that lactate anions in 40 mM concentration depress α_{CO_2} at 38° by 0.0026, which is 0.5 per cent of the solubility in water, an amount that hardly exceeds our experimental error. The actual α_{CO_2} depression caused by addition of lactic acid to serum is presumably less than this: it is the difference between it and the depression caused by the replaced anions, chiefly HCO_3^-. Free lactic acid in amounts greater than those present has no measurable effect on the α_{CO_2} in water solution. We appear justified in considering that the solubility of CO_2 in serum is altered by less than 1 part in 200 by the added lactic acid, and that the α_{CO_2} values in Table VII apply as they stand to serum in its natural state.

The "α^0 saline" values by which the figures in the last columns of Tables VII and VIII were calculated were computed according to the following considerations. The salts in serum acidified with lactic acid consist of approximately 0.160 equivalents of total electrolyte per liter, of which nearly 90 per cent consists of sodium chloride and lactate. As shown by Fig. 3, the effects of NaCl and Na lactate on α^0, the CO_2 solubility per gram of water, are so nearly alike that below 0.16 M concentration the differences hardly exceed the experimental error. It appears therefore that we can with little error estimate the effects of the entire 0.160 equivalents of electrolyte on α^0 to be equal to that of 0.16 M NaCl. From the molar $\Delta\alpha^0$ value, 0.103, of NaCl (Table IV) we calculate the depression of α^0 by 0.16 mols of the salt per kilo of water to be 0.0165 at 38°. However, of the 0.100 mol of lactic acid added about 0.060 remains free, and from the data of Table III this concentration is sufficient to raise α^0 by 0.002 units. The total effect of the salts

and free lactic acid is therefore to depress α^0 by 0.0145. Hence if the solubility of CO_2 per gram of water in serum were affected only by the salts and the added lactic acid, the estimated α^0 would be that of water, 0.5471, reduced by 0.0145, or 0.5326. This is the value assumed for "α^0 saline" in calculating the figures for the last columns of Tables VII and VIII. The average value, 1.038, for the relative solubility ratio, $\frac{\alpha^0 \text{ in serum}}{\alpha^0 \text{ in saline}}$, indicates that the organic constituents of the four sera dissolved on the average enough CO_2 to increase the amount dissolved per gram of water to the extent of 3.8 per cent above what it would be if the organic constituents were inert as CO_2 solvents. It is difficult to estimate the probable error of this calculation, but it appears that we may be justified in stating that the organic constituents of normal serum are responsible for 3.8 ± 1.0 per cent of the CO_2 which the serum can take up by physical solution.

Effects of Lipoids and other Organic Constituents on Solubility of CO_2.

It has been shown above that normal serum and plasma dissolve about 4 per cent more CO_2 than they would if their constituents other than water and salts were inert towards this gas. Stoddard (1926–27) found a similar excess solubility of nitrogen gas in serum, and showed that it was due to the solubility of the gas in the lipoids. We find likewise that the solubility of CO_2 in excess of that attributable to the aqueous saline solution present is due at least in chief part to the solvent effect of the lipoids. This effect is not surprising in view of the fact that oils in general show solubility coefficients for CO_2 higher than that of water. The effect in plasma indicates a solvent power of the lipoids several times that of water. The two lipemic human plasmas and the lipemic serum reported in Table VIII show before extraction with ether α^0 values 10 to 12 per cent above those from Fig. 3 for NaCl solutions of the same salt contents. After extraction with ether the α^0 values were only 2 to 5 per cent above those estimated for saline solutions of similar content. The lipoids were not completely removed by ether extraction, and the excess solubility remaining in the extracted serum is attributable at least in part to the unextracted lipoids. That a part may be attributable to a slight solvent effect of the proteins for CO_2 is not excluded.

However, in comparison with the lipoids, the proteins in the serum act as relatively inert substances. They appear neither to dissolve significant amounts of CO_2 themselves nor to affect the CO_2-dissolving power of the saline water of the plasma. Their chief effect is to decrease the volume of CO_2 dissolved per cc. of serum by displacing water. The proteins thus markedly depress the total CO_2 solubility, α, per cc. of serum, but not the solubility, α^0, per gram of serum water.

In depressing α, the proteins and salts act together, although physically in quite different ways, while the opposing influence of the lipoids is to increase α. Usually the influence of the proteins and salts preponderates; and α, the volume of CO_2 dissolved per volume of fluid, is lower in serum than in water. In our normal sera α averaged 0.510 compared with 0.546 determined by the same methods for water at 38°. In the lipemic sera encountered in some pathological conditions the α values exceed those of normal serum. Thus in Sample 3 of Table VIII the α value is 0.551. It appears that in extreme cases of lipemia, such as are met in nephrosis and severe diabetes, values of α occur which exceed even that of pure water. The low protein content usually encountered in pathologically lipemic sera assists the high fat content in raising the α value.

The organic serum substances other than proteins and lipoids do not appear to affect the solubility of CO_2 to a degree capable of measurement by our methods. The solution of serum proteins, of which the analysis is given in Sample 5 a of Table VIII, behaved quite like serum itself.

Details of Experiments Recorded in Table VIII.—In each case the plasma or serum was acidified with 1 volume per cent of lactic acid of 1.20 specific gravity. The material in Samples 1, 2, and 3 was lipemic plasma and serum obtained for us by Drs. MacKay and Möller from three patients with nephrosis. The plasmas were milky with fats, but low in proteins, in accord with the usual composition of plasmas from nephrotic subjects. A portion of each sample was extracted three times with ether, 2.5 volumes of ether to 1 volume of plasma or serum being used for each extraction. The ether was then removed by bubbling through a rapid current of air. The unextracted control sample was subjected to passage of the same air current, in order to maintain parallel treatment in conditions other than the extraction. The latter removed the visible fat. Plasma Samples 1 and 2 were intended for preliminary experiments, and unfortunately fat determinations were not performed; the entire material was used for the solubility determinations and other analyses.

TABLE VIII.

Effect of Lipoids on Solubility of CO_2 in Plasma and Serum.

Sample of material.	Treatment before acidification.	H_2O content.	Protein content.	Total base.	Lipoids.	α	α^0	α^0 saline estimated.	Relative solubilities. $\frac{\alpha \text{ serum}}{\alpha \text{ water}}$	Relative solubilities. $\frac{\alpha^0 \text{ serum}}{\alpha^0 \text{ water}}$	Relative solubilities. $\frac{\alpha^0 \text{ serum}}{\alpha^0 \text{ saline}}$
		gm. per cc.	*gm. per gm. H_2O*	*eq. per kg. H_2O*	*gm. per gm. H_2O*	*cc. CO_2 per cc. serum*	*cc. CO_2 per gm. H_2O*	*cc. CO_2 per gm. H_2O*			
1. Human lipemic oxalate plasma (nephrosis).	a. None.	0.936	0.035	0.286		0.535	0.574	0.524	0.981	1.049	1.096
	b. Extracted with ether.	0.944	0.037	0.292		0.500	0.532	0.524	0.917	0.973	1.015
2. Human lipemic oxalate plasma (nephrosis).	a. None.	0.934	0.037	0.260		0.542	0.582	0.526	0.993	1.064	1.106
	b. Extracted with ether.	0.946	0.035	0.259		0.510	0.542	0.526	0.935	0.932	1.030
3. Human lipemic serum (nephrosis).	a. None.	0.934	0.039	0.149	0.0316	0.551	0.592	0.531	1.010	1.082	1.115
	b. Extracted with ether.	0.946	0.038	0.152	0.0048	0.520	0.552	0.531	0.953	1.009	1.039
4. Horse serum free from visible fat.	a. None.	0.921	(0.070)	(0.160)	0.0031	0.522	0.569	0.531	0.957	1.040	1.071
	b. Extracted with ether.	0.930	(0.070)	(0.160)	0.0027	0.517	0.559	0.531	0.947	1.022	1.053
5. Solution of serum proteins in 0.15 M NaCl.	a. None.	0.963	0.026	0.156	0.0011	0.530	0.551	0.531	0.972	1.007	1.038
	b. Added olive oil.	0.928	0.025	0.155	0.0356	0.690	0.745	0.531	1.265	1.362	1.403

Figures for total base and protein content of normal horse serum, in parentheses, were not determined by analysis of the serum sample used for this experiment. They are the usual values found previously for blood from the same horse.

Sample 4 was horse serum low in fat content. It was entirely clear, with no visible fat emulsion at all. The content of the invisible lipoids was but slightly affected by ether extraction.

Sample 5 was a solution of serum proteins. The latter were prepared by diluting 400 cc. of serum to 1600 cc. and precipitating by saturation with ammonium sulfate and CO_2. The precipitate was separated by centrifugation, was extracted with ether, redissolved in water, and again precipitated with ammonium sulfate. It was dialyzed in closed collodion sacs in the presence of toluene until free of sulfate. The mixture in the sacs was then brought to 250 cc. volume with sufficient NaCl to make the concentration 0.15 M. Under the influence of the salt the proteins readily redissolved.

One portion of 80 cc. of the solution was acidified with 0.80 cc. of concentrated lactic acid and used for the analyses given for Sample 5 a in Table VIII. Another portion of 65 cc. was mixed with 7 cc. of a 1 : 1 solution of olive oil and petroleum ether. A rapid stream of air was passed through the mixture, simultaneously removing the petroleum ether and emulsifying the oil. A better emulsification was obtained thus than without the initial presence of the petroleum ether. The emulsion thus obtained was acidified with 0.68 cc. of concentrated lactic acid and used for the analyses of Sample 5 b in Table VIII.

Solubility of CO_2 in Diluted Cell Solutions at 38°.

Because of the viscosity of acidified cells, it was necessary, as mentioned above, to use dilute solutions of them, and estimate the solubility in undiluted cells by extrapolation. The cells in weighed amounts were diluted to measured volumes with water, to which had been added amounts of hydrochloric, lactic, or phosphoric acid proportional to the concentration of cells in the first solution, and sufficient to reduce the pH below 3.5. The volume of cells taken in each case was calculated from the weight and the specific gravity. The results of the solubility determination are given in Tables IX and X.

Unlike serum, cells do not permit us to neglect the correction for the anions formed from the added acid. The effects on the quantitatively important electrolytes of the cells of adding an acid, HA, in sufficient amount to depress the pH to 3.0 to 3.5, may be symbolized as follows:

$$(a)\quad B^+Cl^- \rightarrow B^+Cl^-$$

$$(b)\quad B^+HCO_3^- \rightarrow B^+A^- + H_2CO_3$$

$$(c)\quad B^+Hb^- \rightarrow B^+A^- + Hb^+A^-$$

Of these effects only the last (Reaction c) can be important in affecting CO_2 solubility. The chlorides (Reaction *a*) are practically uneffected. The $BHCO_3$ concentration is so small in cells, about 15 mM, that the effect of HCO_3^- replacement by either lactate, $H_2PO_4^-$, or Cl^- anions must be quantitatively negligible. The concentration of lactate$^-$ or $H_2PO_4^-$, anions formed by Reaction *c*, however, is enough to affect measurably the CO_2 solubility. We have corrected for the depressant effect of the Cl^-, lactate$^-$, and $H_2PO_4^-$ anions, formed by addition of the respective acids, by calculating the concentrations of the anions thus formed, and adding to the observed α and α^0 values the $\Delta\alpha$ and $\Delta\alpha^0$ corrections estimated from Figs. 4 and 5. This correction, as seen in Fig. 6, has reduced nearly to the limit of experimental error the differences between results obtained from cells acidified with the different acids.

The correction embodies the assumption that changing the charge of the Hb molecules from negative to positive, as indicated by Reaction *c*, does not alter their influence on α_{CO_2}. This assumption cannot be proved by experiment, for α_{CO_2} cannot be determined in solutions of sufficient alkalinity to permit the existence of BHb, and hence the estimation of the effect of Hb^-. That a considerable change in the electric charge on the Hb molecule does not, however, measurably alter the effect on α_{CO_2} is shown by the agreement between results obtained with cell solutions acidified with lactic acid to pH 3.5 and solutions acidified to pH 2.6 with phosphoric acid.

In the cell solutions to which HCl was added the increase in the Cl anions was assumed to be equal to the HCl added, since dissociation is fairly complete of either the free acid or its salts.

The concentrations of the lactate and acid phosphate anions in the cell solutions were calculated, from the amount of each acid added and the pH of the solution, by means of the familiar Henderson equation for mixtures of weak acids and their salts. The equation may be written as

$$(4) \qquad [BA] \qquad \overline{1 + \frac{H^+}{K'}}$$

where *C* represents the total concentration of buffer, partly in the form of the salt BA, partly in the form of the free acid HA. The concentration of anions has been estimated, on the assumption of complete dissociation of the salt BA, to be equal to [BA]. This same assumption has been made in estimating the effects of the lactate$^-$ and $H_2PO_4^-$ anions on the solubility of CO_2 (Table V and Figs. 4 and 5); whatever error there may be in this assumption is neutralized, in so far as significant error in the present application is concerned, by using the same assumption in the previous determination of the ionic α_{CO_2} depressions.

To determine the factor $\dfrac{1}{1 + \dfrac{H^+}{K'}}$ by which the added acid, *C*, must be

multiplied to estimate the concentration of its anions, it is necessary to know H^+ and K', the apparent dissociation constant of the acid in the buffer mixture. The H^+ values were determined with the hydrogen electrode.

The K' value for the dissociation of the first hydrogen of H_3PO_4 has been estimated by Van Slyke (1922) from hydrogen electrode titrations of Clark and Lubs (1916) to be 1×10^{-2}. The pH of the cell solutions to which H_3PO_4 was added was reduced uniformly to 2.6, corresponding to an H^+ value of 2.5×10^{-3}. Substituting these two values for K' and H^+ in Equation 4 we calculate that 80 per cent of the added H_3PO_4 was converted into the ionized salt, BH_2PO_4.

For lactic acid-lactate mixtures the K' value appears not to have been determined. We have accordingly made an approximate estimation of it at 38° as follows: Equal volumes of 0.2 N lactic acid and 0.1 N sodium hydroxide were mixed. Under these conditions $[BA] = [HA]$ and $H^+ = K'$ (*e.g.* see Van Slyke, 1922). The pH of the solution was determined with the hydrogen electrode, the pH of 0.1 N HCl being taken as 1.08 to standardize the cell. The lactate mixture showed a pH of 3.74, indicating a H^+ activity of 1.8×10^{-4}. This was therefore taken as the K' value for the lactate mixtures in our solutions. It agrees quite closely with the dissociation constant 1.4×10^{-4} found by Ostwald (1889) with his conductivity method.

In lactic acid-cell solutions, Samples 1 a, 2 a, and 3 a (Tables IX and X), the amounts of lactic acid added were proportional to the concentration of cell buffers present, and the pH was uniformly depressed to 3.5, indicating a H^+ value of 3.16×10^{-4}. From this H^+ and the above K' we calculate by Equation 4 that 31 per cent of the added acid was converted into ionized lactate salts. In lactate-cell solutions, Samples 2 a, 2 b, 2 c, 3 a, and 3 b, the proportion of lactic acid per unit of cell concentration was not constant, and the pH was not determined (these were earlier experiments). The proportion of lactic acid per unit of cell concentration, was, however, of a similar order of magnitude, and we have assumed the same pH in calculating the amount of lactate salts formed and the resultant corrections to α and α^0, enclosing the values thus calculated in parentheses to indicate their approximate nature. The solubility corrections due to lactate are so small (0.0026 to α and 0.0012 to α^0) that the error involved in thus approximating the corrections can hardly affect the solubility coefficients by as much as 0.001.

The observed CO_2 solubilities in the cell solutions have been corrected in Tables IX and X, as there indicated, for the added effects of the anions of the added acids and of those portions of the

Solubility Coefficients of CO_2 in Acidified Aqueous Solutions of Ox Cells.

Sample No.	Concentration of cells.	H_2O content of solution at room temperature.	Amount of acid added.	Salts formed from added acid (estimated from pH).	Amount of added acid remaining free (estimated from pH).	$\Delta\ \alpha$ due to salts of added acid.	$\Delta\ \alpha$ due to free acid.	Total α change due to added acid.	α observed.	α corrected for effect of added acid.
Lactic acid added.										
	cc. per 100 cc. solution	*gm. per cc. solution*	*mols per l. solution*	*mols per l. solution*	*mols per l. solution*	*cc. CO_2 per cc. solution*	*cc. CO_2 per cc. solution*	*cc. CO_2 per cc. solution*	*cc. CO_2 per cc. solution*	*cc. CO_2 per cc. solution*
1 a	10.2	0.970	0.084	0.034	0.050	0.0022	0.000	−0.0022	0.536	0.538
b	20.6	0.940	0.180	0.072	0.128	0.0047	0.000	−0.0047	0.527	0.532
c	24.8	0.928	0.270	0.108	0.162	0.0072	0.000	−0.0072	0.520	0.527
2 a	48	0.981	0.100	(0.040)	(0.060)	(0.0026)	0.000	(−0.0026)	0.536	0.539
b	86	0.970	0.100	(0.040)	(0.060)	(0.0026)	0.000	(−0.0026)	0.531	0.534
c	13.4	0.955	0.100	(0.040)	(0.060)	(0.0026)	0.000	(−0.0026)	0.527	0.530
3 a	5.1	0.983	0.100	(0.040)	(0.060)	(0.0026)	0.000	(−0.0026)	0.537	0.540
b	10.2	0.967	0.100	(0.040)	(0.060)	(0.0026)	0.000	(−0.0026)	0.535	0.538
Phosphoric acid added.										
4 a	10.0	0.972	0.078	0.036	0.012	0.0060	0.0006	−0.0066	0.529	0.536
b	19.2	0.948	0.096	0.072	0.027	0.0105	0.0011	−0.0116	0.516	0.528
c	25.6	0.930	0.125	0.095	0.030	0.0135	0.0014	−0.0149	0.503	0.518
Hydrochloric acid added.										
5 a	10.3	0.971	0.050	0.050	0.00	0.0015	0.000	−0.0015	0.531	0.533
b	20.4	0.947	0.100	0.100	0.00	0.0029	0.000	−0.0029	0.526	0.529
Extrapolated α for 100 per cent cells (Fig. 6)										0.45

$\Delta\ \alpha$ indicates depression of α. Figures in parentheses are calculated from estimated pH 3.5, which was not determined in these cases.

TABLE X.

Solubility of CO_2 per Gram of Water in Acidified Aqueous Solution of Ox Cells.

Sample No.	Concentration of cells.	H_2O content of solution at room temperature.	Amount of acid added.	Salts formed from added acid (estimated from pH).	Amount of acid remaining free (estimated from pH).	$\Delta\alpha^0$ due to anions of salts of added acid.	$\Delta\alpha^0$ due to free acid.	Total α change due to added acids.	α^0 observed.	α^0 corrected for effect of added acid.
Lactic acid added.										
	cc. per 100 cc. solution	*gm. per cc. solution*	*mols per kg. H_2O*	*mols per kg. H_2O*	*mols per kg. H_2O*	*cc. CO_2 per gm. H_2O*	*cc. CO_2 per gm. H_2O*	*cc. CO_2 per gm. H_2O*	*cc. CO_2 per gm. H_2O*	*cc. CO_2 per gm. H_2O*
1 a	10.2	0.970	0.088	0.035	0.053	0.0006	−0.0017	+0.0013	0.556	0.555
b	20.6	0.940	0.192	0.076	0.116	0.0014	−0.0037	+0.0023	0.563	0.561
c	24.8	0.928	0.291	0.117	0.174	0.0021	−0.0056	+0.0034	0.563	0.560
	100.0									
2 a	4.8	0.981	0.102	(0.040)	(0.061)	(0.0007)	(−0.0019)	(+0.0012)	0.549	0.548
b	8.6	0.970	0.103	(0.041)	(0.062)	(0.0007)	(−0.0019)	(+0.0012)	0.549	0.548
c	13.4	0.955	0.105	(0.042)	(0.063)	(0.0007)	(−0.0019)	(+0.0013)	0.554	0.553
	100.0									
3 a	5.1	0.983	0.102	(0.041)	(0.061)	(0.0007)	(−0.0019)	(+0.0012)	0.548	0.547
b	10.2	0.967	0.103	(0.041)	(0.062)	(0.0007)	(−0.0019)	(+0.0012)	0.555	0.554
Phosphoric acid added.										
4 a	10.0	0.972	0.049	0.038	0.011	0.0048	0.0002	−0.0050	0.546	0.551
b	19.2	0.948	0.101	0.077	0.024	0.0098	0.0004	−0.0102	0.547	0.557
c	25.6	0.930	0.134	0.102	0.032	0.0130	0.0006	−0.0136	0.543	0.557
Hydrochloric acid added.										
5 a	10.3	0.971	0.052	0.050		0.0010		−0.0010	0.549	0.550
b	20.4	0.947	0.106	0.104		0.0020		−0.0020	0.558	0.560
Extrapolated α^0 for 100 per cent cells (Fig. 6)........										0.60

$\Delta\alpha^0$ indicates depression of α^0. Figures in parentheses are calculated from estimated pH 3.5, which was not determined in these cases.

added acids remaining free. The solubility values thus corrected are given in the last columns of Tables IX and X.

From Fig. 6 it is evident that the corrected CO_2 solubility values change in direct linear proportion to the concentration of cell contents present in the solutions.

The solubility per cc. of solution, represented in the lower curve, decreases as more cells are added. The total effect of the cell

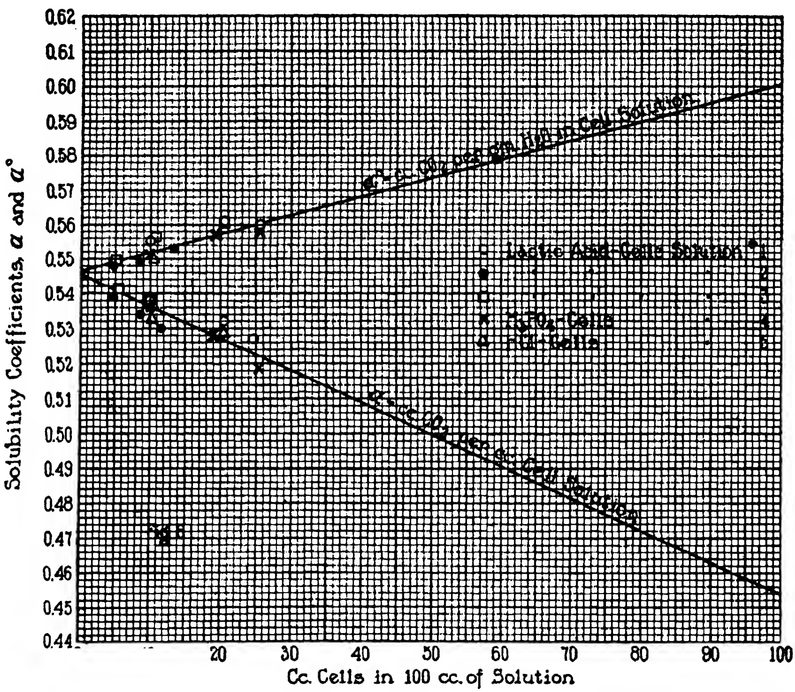

Fig. 6. Solubility of CO_2 at 38° in solutions of ox blood cells in water.

constituents is therefore to depress the solvent power per unit volume of solution.

However, the rising slope of the upper curve indicates that cell contents, like serum contents, increase the amount of CO_2 dissolved *per gram of water* in the solutions. Some organic constituent or constituents of the cells therefore act as solvents for CO_2, and their effect on the amount dissolved per gram of

water more than offsets the depressant effect of the mineral salts present. Whether in the cells, as in serum, the lipoids are responsible for the CO_2 dissolved by the organic constituents, or whether the proteins also act to an appreciable extent as CO_2 solvents, we have not been able to settle.

In order to estimate the solubilities in the undiluted cells we have extended the straight lines, representing α and α^0 in Fig. 6, until they indicate at 100 the extrapolated coefficients for the cells themselves. Such extrapolation involves the assumption that the direct linear relationship, between the concentration of cell constituents present and their effect on the solubility of CO_2, found to hold for solutions containing up to 25 per cent by volume of cells, holds also for the remaining range up to 100 per cent. It has been

TABLE XI.

Analyses of Ox Cells Used for Solubility Experiments Reported in Tables IX and X.

Sample No.	H_2O content of cells.	K content.	Na content.	Chloride content.	Fat content (Röse-Gottlieb).	Protein content (N × 5.78).
	kg. per l.	*m.-eq. per l.*	*m.-eq. per l.*	*m.-eq. per l.*	*gm. per l.*	*gm. per l.*
1	0.718	27.3	81.6	65.8	1.90	287
2	0.669	29.9	90.9	72.1	1.22	306
3	0.716	30.1	91.7	72.2	2.72	291
4	0.743	34.0	102.1	81.8	1.17	304
5	0.749	32.0	97.0	56.6	0.84	280

impossible to prove this assumption, because of the viscosity of the more concentrated cell solutions. There is however, no reason to expect significant deviation from the linear relationship. The electrolyte content, indicated by the total base, reaches in cells only about 170 milli-equivalents per kilo of water present (Van Slyke, Wu, and McLean, 1923), and the curves of Figs. 2 and 3 show hardly perceptible deviations from straight lines below this concentration. That the effect of the non-electrolytes, in the relatively small molar concentration present, would be other than linear, appears improbable.

The extrapolation of the upper curve of Fig. 6 indicates that cells saturated with CO_2 at atmospheric pressure and 38° dissolve 0.60 cc. of CO_2, measured under standard conditions, per gram of water present. The probable error of the extrapolation appears

to be about 0.01 cc. The ordinary Bunsen solubility coefficient, plotted in the lower curve of Fig. 6, indicates by extrapolation an α value for the ox cells used of 0.45 cc. of CO_2 per cc. of cells. The ox cells contained varying proportions of water (see Table XI). Human cells average about 0.73 gm. of water per cc. (unpublished data) and, with the same solvent power as ox cells per gram of water, would have a Bunsen solubility coefficient of 0.44.

TABLE XII.

Solubility of CO_2 in Cell Solutions and Serum Saturated at Low and High CO_2 Pressures.

Solution analyzed.	CO_2 tension at 38°.	CO_2 content at 22.0–22.5°.	α	α^0
	mm.	*vol. per cent*	*cc. CO_2 per cc. solution*	*cc. CO_2 per gm. H_2O*
50 cc. horse cells + 150 cc. H_2O + 20 cc. 1.0 N HCl.				
H_2O = 0.928 gm. per cc. at 22.5°.	709.7	48.50	0.517	0.560
Protein = 0.085 gm. per cc.		48.61	0.518	0.561
Total base = 34.6 m.-eq. per kg. H_2O.	60.2	4.14	0.520	0.563
pH = 3.7.		4.12	0.517	0.561
100 cc. horse serum + 10 cc. 1.0 N HCl.	709	49.15	0.524	0.561
H_2O = 0.939 gm. per cc. at 25°.		49.45	0.527	0.564
pH = 3.2.	41.9	2.908	0.525	0.562
		2.900	0.523	0.560
	42.4	2.900	0.517	0.554
		2.925	0.522	0.559

Solubility of CO_2 in Serum and Cells at Low CO_2 Tensions.

Conant and Scott (1926) recently demonstrated that when a solution of hemoglobin was equilibrated with nitrogen gas at different partial pressures, the amount absorbed per gram of water exceeded the amount absorbed by water in the absence of other substances. The excess of absorbed N_2 taken up by the hemoglobin did not, in its variations with varying N_2 tension, follow Henry's law. It followed the Freundlich adsorption equation:

(5) $$\text{Amount adsorbed} = a\,p^b$$

where p is the partial pressure of gas, and a and b are constants for a given solution, the usual value of b being 0.5 or less.

If significant amounts of the CO_2 were held by adsorption on the colloid particles or molecules one would expect to find higher solubility coefficients at low than at high tensions, as did Conant and Scott in the case of nitrogen. We therefore determined the solubility of CO_2 in acidified serum and cell solutions at CO_2 pressures of 40 to 50 mm. as well as at 1 atmosphere. The results given in Table XII show that at the lower CO_2 tensions the same solubility coefficient was obtained as at 1 atmosphere. Within the limits of CO_2 pressures under which our experiments were carried out, CO_2 is dissolved by both plasma and cells in accordance with Henry's law. There is no evidence of deviation from it such as was found by Conant and Scott in the case of nitrogen gas.

Effect of Change in α_{CO_2} on the Calculation of Serum pH by Hasselbalch's Equation.

From the following considerations it is evident that changing the value of α from Bohr's 0.541 to our 0.510 necessitates *no revision* of the serum pH values in the literature that have been calculated by Hasselbalch's equation, with the Bohr α.

The concentration of "physically dissolved CO_2," symbolized as $[H_2CO_3]$, is estimated, by definition[1] of the Bunsen solubility coefficient, α, as

$$(6) \qquad \left.\begin{array}{l}[H_2CO_3] \text{ in cc. } CO_2 \\ \text{dissolved per cc.} \\ \text{of solution}\end{array}\right\} = \alpha \times (\text{atmospheres } CO_2 \text{ pressure})$$

$$= \alpha \times \frac{\text{mm. } CO_2 \text{ pressure}}{760}$$

$$\frac{\alpha p}{760}$$

where p expresses CO_2 pressure in mm. of mercury. Hence

$$(7) \qquad \left.\begin{array}{l}[H_2CO_3] \text{ in cc. } CO_2 \\ \text{dissolved per liter} \\ \text{of solution}\end{array}\right\} = 1000 \frac{\alpha p}{760} = \frac{\alpha p}{0.76}$$

According to Guye and Pintza (1908) 1 mg. molecule of CO_2 gas at 0°, 760 mm., occupies 22.26 cc. of volume. Hence the concentration in mM per liter is calculated from the concentration in cc. per liter by dividing the latter by 22.26.

$$(8) \qquad [H_2CO_3] \text{ in mM per liter} = \frac{\alpha\, p}{22.26 \times 0.76} = 0.0591\, \alpha\, p$$

Hasselbalch's equation may be written

$$(9) \qquad \begin{aligned} pH &= pK' + \log [BHCO_3] - \log [H_2CO_3] \\ &= pK' + \log [BHCO_3] - \log (0.0591\, \alpha\, p) \\ &= pK' + \log [BHCO_3] - \log 0.0591 - \log \alpha - \log p \end{aligned}$$

It is obvious that any change in α affects pH to the same extent that it affects log α, and in the opposite direction. Increasing log α by 0.1 will decrease pH by 0.1, and *vice versa*, if the same pK′ is used.

Hence decreasing α from 0.541 to 0.510 would increase the pH, calculated by Hasselbalch's equation, by 0.026, which is the difference between log 0.541 and log 0.510.

However, if α were thus changed, one would have to revise the pK′ value used, by recalculating it from the original values of pH, p, and $[BHCO_3]$, from which the pK′ values in past use have been calculated. pK′ is calculated from experimentally determined pH, $[BHCO_3]$, and p, as:

$$(10) \qquad pK' = pH - \log [BHCO_3] + \log 0.0591 + \log \alpha + \log p$$

Hence any increase in log α would cause an equal increase in the pK′ estimated from identical data. The revision of pK′ would neutralize the revision of α, and determinations of pH from $[BHCO_3]$ and p values with *both* α and pK′ thus corrected would, by the Hasselbalch equation, yield the same pH values as with the old constants.

There would, it is true, be a slight effect on the calculated pH, when $[BHCO_3]$ is determined in the manner usually employed. $[BHCO_3]$ has usually been estimated as the difference between the total CO_2 in solution, found by analysis, and H_2CO_3, calculated from p and α.

$$(11) \qquad \begin{aligned} [BHCO_3] &= [\text{total } CO_2] - [H_2CO_3] \\ &= [\text{total } CO_2] - 0.0591\, \alpha\, p \end{aligned}$$

Hence any change in α does affect [$BHCO_3$]. However, at physiological blood pH ranges, [H_2CO_3] is only about one-twentieth as great as [$BHCO_3$]. Consequently such change in α as we are concerned with causes only a negligible percentage change in [$BHCO_3$], and a correspondingly negligible effect on the calculated pH. For example, at pH 7.4, with [$BHCO_3$] 20-fold as great as H_2CO_3 a change of α from 0.541 to 0.510, lowering α by 5.7 per cent, would affect [$BHCO_3$] by only $\frac{5.7}{20} = 0.28$ per cent, and the resultant alteration in calculated pH would be only 0.001, a change within the limits of error of present measurements.

It is therefore obvious that our revision of α_{CO_2} for blood serum involves no necessity for recalculating serum pH values in the literature that have been calculated by Hasselbalch's equation.

If, on the other hand, one changes from the use of Bohr's α, 0.541, to our lower α, 0.510, one must make a corresponding change in pK′, lowering it by 0.026 units. Otherwise the pH values calculated with our α would be 0.026 units too high.

SUMMARY.

The measurable deviations of the solubility of CO_2 in serum from its solubility in water have been found due to the following factors: *salts*, depressing the solubility about 3 per cent by lowering the solvent power of the water for CO_2, and to a lesser extent by displacing a small amount of water; *proteins*, depressing the solubility of CO_2 per cc. of serum several per cent by displacing water; *lipoids*, raising the solubility (by about 4 per cent in normal serum) because of their own high solvent power for CO_2. The combined effect is to reduce the solubility in normal serum to 93 to 94 per cent of its solubility in water. But in lipemic serum the solubility may exceed that in water.

The values for blood cells were obtained by extrapolation from determinations on aqueous solutions of the cells. Hence the coefficients for cells are not significant to more than the second place.

The same solubility coefficient was found for serum whether saturated at 1 or $\frac{1}{4}$ atmosphere of CO_2 pressure. Similar independence of the pressure was found for the CO_2 solubility coefficient of a solution of red blood cells. There was no evidence

of deviation from Henry's law, such as might have been expected if any of the colloids of either serum or cells took up significant amounts of CO_2 by adsorption.

The following solubility coefficients for CO_2 in acidified solutions at 38° have been found.

	Water.	Normal human serum, average.	Lipemic human serum, maximum.	Ox blood cells, average.
α = cc. CO_2, measured under standard conditions, dissolved per cc. fluid under 760 mm. CO_2 pressure.................	0.5455 ±0.001	0.510	0.552	0.44*
Relative α, water α being 100............	100.0	93.4	101.2	79
α^0 = cc. CO_2, measured under standard conditions, dissolved per gm. water in solution..............................	0.5471 ±0.001	0.553	0.592	0.60
Relative α^0, water α^0 being 100..........	100.0	101.4	108.2	110
Factor $\frac{\alpha}{7.6}$, for formula, $[H_2CO_3]$ in vol. per cent $CO_2 = \frac{\alpha}{7.6} \times p$..............	0.0718	0.0671	0.0726	0.0566
Factor $\frac{\alpha}{22.26 \times 0.76}$, for formula, $[H_2CO_3]$ in mM per l. $= \frac{\alpha}{22.26 \times 0.76} \times p$.....	0.03222	0.0301	0.0326	0.0254
Factor $\frac{\alpha^0}{22.26 \times 0.76}$, for formula, $[H_2CO_3]$ in mM per kg. $H_2O = \frac{\alpha^0}{22.26 \times 0.76} \times p$.	0.03232	0.0327	0.0350	0.0354

* For cells with 0.73 gm. H_2O per cc.

In water solutions of the chlorides, lactates, and acid phosphates of potassium and sodium, the depressions of α and α^0 per mol of electrolyte have been found to be additive functions of the depressions due to the individual ions. In descending order of their effect on α^0 the ions rank as follows: $H_2PO_4^- > Na^+ > K^+ > Cl^- >$ lactate$^- > HC_2O_4^- > H^+$.

Revision of the solubility coefficient of serum from the α value 0.541, estimated by Bohr (1905) to the value 0.510 found by us, does not necessitate revision of the serum pH values in the literature that have been calculated by Hasselbalch's equation. The lowering of α is compensated by the lowering of 0.026 units which occurs in the pK′ of Hasselbalch's equation, when the pK′ is recalculated from the same experimental data with our lower α value.

BIBLIOGRAPHY.

Austin, J. H., Cullen, G. E., Hastings, A. B., McLean, F. C., Peters, J. P., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liv, 121.

Bohr, C., *Skand. Arch. Physiol.*, 1905, xvii, 104.

Bohr, C., *Wied. Ann. Physik u. Chem.*, 1891, xliv, 44; 1897, lxii, 644; 1899, lxviii, 500.

Bohr, C., and Bock, J., *Wied. Ann. Physik u. Chem.*, 1891, [2] xliv, 318.

Buytendyk, F. J., Brinkman, R., and Mook, H. W., *Biochem. J.*, 1927, xxi, 576.

Clark, W. M., and Lubs, H. A., *J. Biol. Chem.*, 1916, xxv, 479.

Conant, J. B., and Scott, N. D., *J. Biol. Chem.*, 1926, lxviii, 107.

Fahr, G., *J. Physiol.*, 1912, xliii, 417.

Geffcken, G., *Z. physik. Chem.*, 1904, xlix, 271.

Guye, P. A., and Pintza, A., *Mem. Soc. Physique et Hist. Nat. Genève*, 1908, xxxv, 569.

Henderson, Y., *J. Biol. Chem.*, 1918, xxxiii, 31.

Just, G., *Z. physik. Chem.*, 1901, xxxvii, 342.

Ostwald, W., *Z. physik. Chem.*, 1889, iii, 173.

O'Brien, H. R., and Parker, W. L., *J. Biol. Chem.*, 1922, l, 289.

Schoen, R., *Z. physiol. Chem.*, 1923, cxxvii, 243.

Setschenow, J. M., *Ann. chim. et physique*, 1892, xxv, 226.

Siebeck, R., *Skand. Arch. Physiol.*, 1909, xxi, 268.

Stoddard, J. L., *J. Biol. Chem.*, 1926–27, lxxi, 629.

Van Slyke, D. D., *J. Biol. Chem.*, 1922, lii, 551; 1923–24, lviii, 523.

Van Slyke, D. D., Hiller, A., and Berthelsen, K. C., *J. Biol. Chem.*, 1927, lxxiv, 659.

Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, lxi, 523.

Van Slyke, D. D., and Sendroy, J., Jr., *J. Biol. Chem.*, 1927, lxxiii, 127.

Van Slyke, D. D., Wu, H., and McLean, F. C., *J. Biol. Chem.*, 1923, lvi, 765.

STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD.

XI. THE SOLUBILITY OF HYDROGEN AT 38° IN BLOOD SERUM AND CELLS.

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Fahr's (1911–12) determinations of the solubility of hydrogen at 29.7° appear to be the only ones in the literature in which the solvent powers of blood cells and serum for this gas have been determined. Hydrogen is occasionally used as an inert gas in physiological work (*e.g.* the lung volume determination of Van Slyke and Binger (1923), Lundsgaard and Schierbeck (1923)). A knowledge of the solubility of the gas in blood serum and cells at body temperature seems desirable. Hence we present the accompanying data, which were originally obtained for a specific application.

The serum and cells used were obtained by centrifugation of defibrinated ox blood.

The saturation, with hydrogen gas from a metal tank, was performed in a rotating double-chambered saturating vessel according to the "First Saturation Method" of Austin *et al.* (1922). The hydrogen atmosphere was renewed twice during the saturation, which was prolonged to an hour.

The H_2 content of the liquid phase was determined in a Van Slyke-Neill (1924) manometric apparatus with a chamber of 100 cc. capacity and a calibration mark at 1.000 cc. for measurement of the volume of extracted H_2 at the time of its pressure reading. Samples of 15 cc. each of saturated cells or serum were transferred from the smaller chamber of the saturating vessel to the manometric apparatus by means of the special pipette described in section XII of Van Slyke and Neill's paper (1924). The H_2 gas

was extracted from the fluid by 3 minutes shaking in the evacuated chamber. To remove CO_2 and possible traces of O_2 from the extracted gas, the latter was transferred to the special Hempel pipette described by Van Slyke and Hiller ((1928), see Fig. 2), where the gas was shaken with alkaline sodium hydrosulfite solution.

Before the H_2 was returned to the chamber for measurement the serum or cells were washed out of the chamber and replaced by 15 cc. of water, so that the gas volume at the final reading could be taken over a transparent fluid with a perfectly sharp meniscus. The air in the water was extracted and ejected, and the manometer reading p_0 was taken with the meniscus of the solution at the 1 cc. mark in the gas-free chamber.

The hydrogen gas from the Hempel pipette was then returned to the chamber, and the manometer reading p_1 was taken with the meniscus again at the 1 cc. mark. The hydrogen content of the analyzed fluid was calculated as

$$\text{Volume per cent } H_2 = (p_1 - p_0) \times \text{factor}$$

The factor in the above equation was computed by the formula given on p. 542 of Van Slyke and Neill's paper. The α'_{H_2} values used in the formula were from Winkler's data in the Landolt-Börnstein "Tabellen." The i factor was taken as 1.000.

The hydrogen gas from 15 cc. of saturated cells or serum was sufficient to give at 1 cc. volume a pressure of approximately 170 mm., duplicate determinations at the same temperature being constant to within 1 mm.

The H_2 with which the serum had come to equilibrium contained slight amounts of CO_2; when cells had been saturated there was also a little oxygen extracted from them, and a slight amount was present as impurity in the hydrogen used. The amounts of these gases mixed with the H_2 were determined with Henderson's modification (1918) of Haldane's apparatus for analysis of expired air. The residue of gas not absorbed by KOH or pyrogallol was considered to be H_2. The pressure, p_{H_2}, of hydrogen, under which the cells or serum was saturated, was estimated as

$$p_{H_2} = \frac{\text{per cent } H_2 \text{ in tonometer gas}}{100} \times (B - 49.7)$$

where B represents barometric pressure in mm. of mercury, and 49.7 is the vapor tension of water at 38°.

The value of the Bunsen solubility coefficient, α, was calculated by the equation

$$\alpha = \frac{760}{p} \times \frac{[H_2]}{100} \times \frac{V_R}{V_{38°}} = \frac{756}{p} \times \frac{[H_2]}{100}$$

where p represents H_2 pressure at the time of saturation, calculated as above, $[H_2]$ is the volume per cent of hydrogen in solution, V_R and $V_{38°}$ represent respectively the volumes occupied by 1 gm. of the solution at room temperature and at 38°. The equation has been discussed in the accompanying paper on CO_2 solubility (Van Slyke, Sendroy, Hastings, and Neill (1928)). The H_2 determinations in the saturated fluids were all performed at a room temperature near 22° where the factor $\frac{V_R}{V_{38°}}$ is 0.995, and $\frac{V_R}{V_{38°}} \times 760 = 756$.

Our value of 0.01620 for α_{H_2} in water at 38° is 2.0 per cent below the value for that temperature interpolated from Winkler's data in the Landolt-Börnstein "Tabellen."

Fahr's (1911–12) value at 29.7° is 4 per cent lower than that interpolated from Winkler's data for that temperature. Our value for α_{H_2} in water accordingly lies, as nearly as one can estimate by interpolation, between Fahr's and Winkler's.

For ox serum at 38° our relative solubility, $\frac{\alpha \text{ observed}}{\alpha \text{ in } H_2O}$, of 0.946 is practically identical with Fahr's 0.950 at 29.7°.

In cells Fahr found relative H_2 solubility values at 29.7° from 0.86 to 0.90; ours at 38° are 0.891 and 0.904.

The α^0 values indicate cc. of H_2 dissolved per gram of water in solution. From these it is obvious that serum and cells, particularly the latter, dissolve more hydrogen than would be taken up by the water in them if the other constituents were inert. The values of the ratio $\frac{\alpha^0 \text{ observed}}{\alpha^0 \text{ in water}}$ show that serum dissolved 2.2 per cent more of the gas, and cells 18.8 per cent more, than the water present would by itself dissolve. The organic constituents

evidently dissolve considerable amounts. When we consider the depressant effect of the alkali salts on the solubility, the solvent power of the organic constituents stands out even more pronouncedly. In the last column of Table I the observed α^0 values

TABLE I.

Solubility of Hydrogen in Water, Serum, and Blood Cells at 38°.

Material.	H_2O content at 22°.	H_2 tension at 38°.	H_2 content (solution at 22° when analyzed).	α_{H_2} H_2 per cc. solution at 38°.	$\alpha^0_{H_2}$ H_2 per gm. H_2O in solution at 38°.	Relative solubilities. $\frac{\alpha \text{ observed}}{\alpha H_2O}$.	Relative solubilities. $\frac{\alpha^0 \text{ observed}}{\alpha^0 H_2O}$.	Relative solubilities. $\frac{\alpha^0 \text{ observed}}{\alpha^0 \text{ saline}}$.
	gm. per cc.	*mm.*	*vol. per cent*	*cc.*	*cc.*			
H_2O	0.998	707	1.508	0.01613				
"	0.998	702	1.508	0.01624				
"	0.998	702	1.498	0.01613				
"	0.998	689	1.479	0.01623				
"	0.998	699	1.492	0.01614				
"	0.998	699	1.518	0.01642				
"	0.998	708	1.499	0.01601				
"	0.998	698	1.498	0.01622				
"	0.998	698	1.507	0.01632				
Average				0.01620	0.01632	1.000	1.000	1.036
0.15 M NaCl.	0.995	706	1.460	0.01563				
	0.995	706	1.452	0.01555				
Average				0.01559	0.01576	0.963	0.966	1.000
Ox serum.	0.924	694	1.403	0.01528	0.01662	0.943	1.018	
	0.924	694	1.413	0.01539	0.01676	0.950	1.026	
Average				0.01533	0.01669	0.946	1.022	1.058
Ox cells.	0.750	693	1.323	0.01443	0.01925	0.891	1.179	1.234
	0.750	693	1.342	0.01464	0.01952	0.904	1.196	1.252
Average				0.01454	0.01938	0.898	1.188	1.241

are compared with the α^0 values for the amount of physiological saline present. It there becomes apparent that the organic constituents increase the hydrogen taken up by serum by about 6 per cent, and that taken up by cells by about 24 per cent.

Stoddard (1926–27) has noted a similar behavior of the organic constituents toward nitrogen, and Van Slyke, Sendroy, Hastings, and Neill (1928) have observed it towards CO_2. The organic solvents for N_2 and CO_2 in the serum were identified as the lipoids, but the data of the above authors did not indicate in cells whether the lipoids or proteins or both were acting as gas solvents.

SUMMARY.

Ox serum was found to dissolve, per unit volume at 38°, 96.3 per cent as much hydrogen, and ox cells 89.8 per cent as much, as is dissolved by water. Per gram of water present, serum dissolved 6 per cent more, and cells 24 per cent more, than is dissolved per gram of water in 0.15 M NaCl solution. The organic constituents therefore dissolve considerable amounts of hydrogen.

BIBLIOGRAPHY.

Austin, J. H., Cullen, G. E., Hastings, A. B., McLean, F. C., Peters, J. P., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liv, 121.

Fahr, G., *J. Physiol.*, 1911–12, xliii, 417.

Henderson, Y., *J. Biol. Chem.*, 1918, xxxiii, 31.

Lundsgaard, C., and Schierbeck, K., *Am. J. Physiol.*, 1923, lxiv, 210.

Stoddard, J. L., *J. Biol. Chem.*, 1926–27, lxxi, 629.

Van Slyke, D. D., and Binger, C. A. L., *J. Exp. Med.*, 1923, xxxvii, 457.

Van Slyke, D. D., and Hiller, A., *J. Biol. Chem.*, 1928, lxxviii, 807.

Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, lxi, 523.

Van Slyke, D. D., Sendroy, J., Jr., Hastings, A. B., and Neill, J. M., *J. Biol. Chem.*, 1928, lxxviii, 765.

GASOMETRIC DETERMINATION OF HEMOGLOBIN BY THE CARBON MONOXIDE CAPACITY METHOD.

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The method here described is based on the capacity of hemoglobin to combine with the same maximum volume of CO as of O_2. The blood sample, which may vary from 0.1 to 2.0 cc., is saturated with CO in the 50 cc. chamber of the manometric blood gas apparatus of Van Slyke and Neill (1, 2). Approximately 2 cc. of carbon monoxide are introduced with the blood, and the chamber is evacuated and shaken. The carbon monoxide tension of approximately 25 mm. is sufficient to change the hemoglobin quantitatively to HbCO, while O_2 and N_2 are at the same time completely extracted from the blood. The gases are ejected, and the HbCO is determined by measurement of the CO set free by acid ferricyanide solution.

The corrections for physically dissolved O_2 and N_2 are eliminated by the above procedure. The substituted correction for physically dissolved CO is only 0.3 volume per cent, so that variations in it are negligible.

Economy of material and apparatus is attained by performing both the saturation with CO and its subsequent analysis in the chamber of the gas apparatus. The loss of part of the blood sample entailed in the usual oxygen capacity determination (1, 3, 4) by transfer after saturation in a separate vessel, is thus avoided. Consequently gasometric hemoglobin determinations are made possible with 0.1 or 0.2 cc. of blood.

Reagents.

Acid Ferricyanide Solution.—To 92 volumes of a stock solution containing 32 gm. of $K_3Fe(CN)_6$ per 100 cc. are added 8 volumes

of concentrated lactic acid, of specific gravity 1.2. This concentrated acidified solution undergoes very slow decomposition, but can be used for over 2 months.[1]

Air-Free 1 N Sodium Hydroxide Solution.—A portion of this solution is made air-free (see p. 534 of Van Slyke and Neill (1)) each day and kept under oil, or is prepared less frequently and confined over mercury in the modified Hempel pipette shown in Fig. 1. For this purpose mercury replaces the water shown in Fig. 1, and the air-free solution replaces the CO gas.

5 N Sodium Hydroxide.—An approximately CO_2-free, saturated (18 N) NaOH solution is prepared by the usual procedure of dissolving NaOH in an equal weight of water, and permitting the solution to stand until the carbonate has settled. 27 cc. are diluted to 100 cc. to make the 5 N solution, which is kept protected from atmospheric CO_2 in the soda-lime tube, described previously (5).

Carbon Monoxide Gas.—This is prepared by warming a mixture of anhydrous formic and sulfuric acids. According to the reaction, $HCOOH = CO + H_2O$, each cc. of formic acid yields about 500 cc. of CO. In Fig. 2 is shown a simple arrangement for preparing and storing 3 liters of the gas, enough for 1500 analyses. The two 5 liter aspirator bottles are connected by a rubber tube, the bore of which should be as wide as 15 mm. in order to assure a flow of water sufficiently free to prevent the development of back pressure during generation of the CO. Before the latter process is begun the bottle, *A*, is filled completely with water. Sulfuric acid is dropped slowly into the formic acid in the tube, which is gently warmed with a micro burner. When about 300 cc. of mixed air and CO from the test-tube have collected in *A* the gases are ejected by opening the outlet cock of *A* and raising *B*. Then the rest of the sulfuric acid is run slowly into the formic acid, and the reaction is continued until the CO from all the formic acid has been

[1] In a portion of the acid ferricyanide solution, kept at room temperature in a flask of ordinary white glass and exposed to diffuse but not direct sunlight, the ferricyanide content was determined at intervals by measuring the N_2 gas evolved by the reaction with alkaline hydrazine solution. $4K_3Fe(CN)_6 + 4KOH + N_2H_4 = 4K_4Fe(CN)_6 + 4H_2O + N_2$. The percentages of original ferricyanide found were: 7 days, 98 per cent; 10 days, 93 per cent; 21 days, 92.3 per cent; 60 days, 91 per cent.

collected in A. The rubber tube connecting the test-tube with A is then closed with a screw clamp close to the glass inlet tube of A, and the test-tube is disconnected from A. *Because of the toxicity of CO the above operation should be carried out in a hood or where there is a free draft of air.*

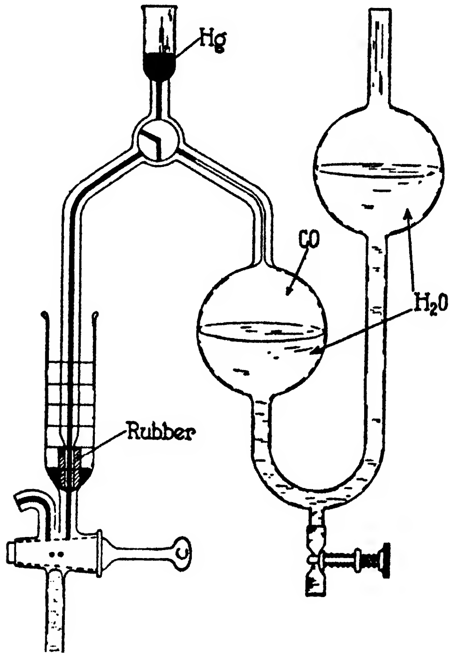

FIG. 1. Hempel pipette provided with 3-way stop-cock. The bulbs are of about 50 cc. capacity each. The capillary is of 1 mm. bore. When the pipette is not in use a little mercury is let into the capillary leading to the CO bulb, to seal the 3-way cock and prevent leakage around it. This drop of mercury in the capillary to the right of the cock is shown in the figure. This pipette may also be used to store air-free solutions. In this case the solution replaces the CO gas shown above, and mercury is used where water is indicated in the above figure. Supplied by the makers of the Van Slyke-Neill apparatus (1).

Procedure for Analysis of 2 Cc. Blood Samples.

Measurement of Blood Sample into Apparatus.—1 drop of caprylic alcohol is drawn into the capillary beneath the cup of the

manometric apparatus. (This capillary should be of only 1 mm. bore.) Into the cup are measured 4.75 cc. of water. With a stop-cock pipette, provided with a rubber tip (see Fig. 4, p. 532, of Van Slyke and Neill (1)), 2 cc. of blood are run directly into the chamber followed by the 4.75 cc. of water.

Measurement of CO into Chamber of Apparatus.—Approximately 2 cc. of CO, measured at atmospheric pressure, are required. This amount provides 0.50 cc. to combine with the maximum amount of hemoglobin ordinarily found in human

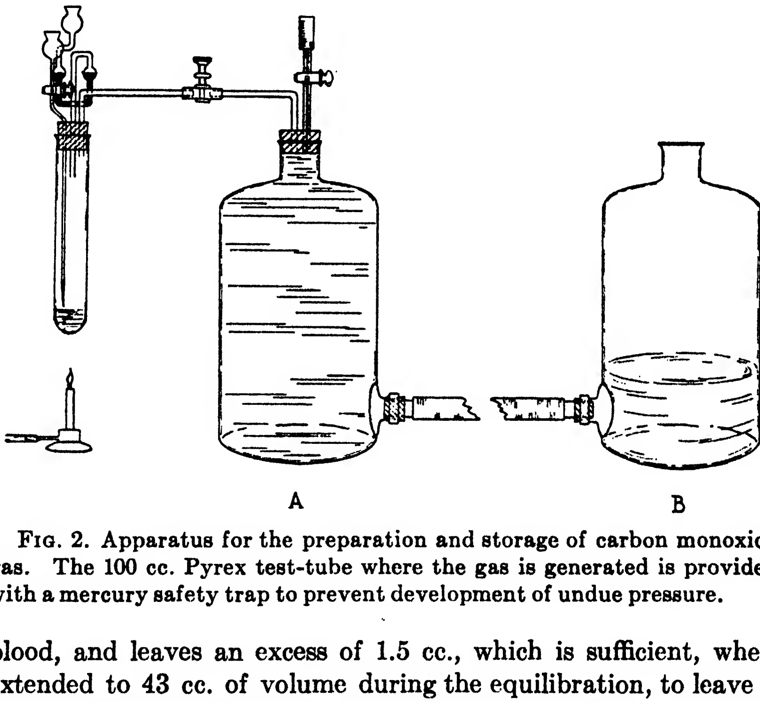

Fig. 2. Apparatus for the preparation and storage of carbon monoxide gas. The 100 cc. Pyrex test-tube where the gas is generated is provided with a mercury safety trap to prevent development of undue pressure.

blood, and leaves an excess of 1.5 cc., which is sufficient, when extended to 43 cc. of volume during the equilibration, to leave a CO pressure of about 25 mm. and insure quantitative change of Hb to HbCO. The exact amount of CO may vary from 2 cc. to 3 cc. if it is accurately duplicated in the blank analyses in which the correction for physically dissolved gases is determined. Any convenient device may be used for measuring the CO. The following has been found satisfactory.

From the Hempel pipette 2 cc. of the CO are measured into the chamber of the Van Slyke-Neill apparatus by the following pro-

cedure. 1 or 2 cc. of mercury are placed in the cup above the chamber. The outlet capillary of the Hempel pipette is filled with mercury. The tip of the Hempel pipette is then fitted into the bottom of the cup, as shown in Fig. 1. The two cocks shown in Fig. 1 are now turned so that the CO gas can flow from the Hempel pipette into the chamber of the Van Slyke-Neill apparatus. The flow is regulated, not by either of the cocks shown, but by the cock leading to the mercury leveling bulb of the manometric apparatus. With the leveling bulb in the mid-position shown in Fig. 1 of Van Slyke and Neill's paper, this cock is opened slowly, and mercury is withdrawn from the chamber until CO gas has entered it as far as the 2 cc. mark. The gas is thus measured under a pressure indicated by the difference in height between the water columns in the two bulbs of the Hempel pipette. This difference may vary from about 10 cm. of water when the pipette is full of gas to 0 cm. when it is nearly empty. Such variation, however, amounts to only 0.01 of an atmosphere, which is negligible in its effect on the 0.30 volume per cent correction for dissolved CO.

Equilibration of Blood Solution with CO.—Both blood solution and CO gas having been measured into the chamber, the cock of the latter is closed and is sealed with a drop of mercury, and the chamber is evacuated. With the mercury meniscus at the 50 cc. mark, the chamber is shaken until equilibrium is reached. The time required varies somewhat with the speed of the motor. The necessary time is found by equilibrating for different periods in the blank analyses described below, and finding how long one must shake the chamber in order to reduce the blank to a minimum. In our apparatus this time is 1 minute. Equilibration being completed, the mixture of free gases, composed of all the O_2 and N_2, and part of the CO_2 from the blood, together with the excess CO, is ejected from the chamber.[2]

Determination of CO Bound as HbCO.—After ejection of the free gases, the chamber is evacuated until the blood solution is in the lower fourth, in order to keep the calibrated upper portion clean when the acid ferricyanide to be added precipitates methemoglobin. About 1 cc. of mercury and 2 or 3 cc. of water are placed

[2] For technique of ejection of gas see (6) foot-note 3, p. 240.

in the cup. Through the mercury seal 0.25 cc. of the acid ferricyanide solution is added from a micro burette graduated into 0.01 cc. divisions, and provided with a rubber-tipped delivery capillary.[3] Before the tip of the burette is inserted into the mercury it is moved through the supernatant layer of water, which dislodges any adherent ferricyanide crystals or air bubbles.

After addition of the ferricyanide, only enough mercury is admitted into the capillary beneath the cup to fill that capillary and the bore of the cock. It is preferable to have no mercury globules run down into the chamber at this time, because they are likely to adhere to the film of ferricyanide on the walls of the upper 2 cc. of space, which it is desirable to keep free and clean for subsequent gas measurement.

To extract the CO set free from HbCO by action of the ferricyanide, the mercury in the chamber is lowered to the 50 cc. mark, and the chamber is shaken slowly for about 5 seconds, to avoid too rapid foaming, then vigorously for 3 minutes.

Mercury is now admitted from the leveling bulb into the chamber until the gas space above the solution is reduced to 5 or 6 cc., and the CO_2 in the gas phase is absorbed with 1 cc. of air-free 1 N NaOH in the manner described on pp. 545–546 of Van Slyke and Neill (1).

The volume of the remaining gas, CO, with a slight trace of air introduced with the ferricyanide, is brought to 2 cc., and the pressure observed on the manometer is recorded as p_1. The gas is ejected.[3] The stop-cock of the chamber is again sealed with a drop of mercury, and the fluid meniscus is lowered to the 2 cc. mark. The manometer reading now observed, with the chamber gas-free, is recorded as p_2.

The hemoglobin content of the blood in terms of CO- or O_2-binding capacity is calculated by the equation

$$\text{CO or } O_2 \text{ capacity} = (p_1 - p_2 - c)f$$

f is a factor from Table II or III of Van Slyke and Neill (1) and c is a correction, determined by blank analysis, for the slight amounts of CO physically dissolved by the equilibrated blood solution, and of air admitted in solution in the 0.25 cc. of ferricyanide.

[3] Shown in Fig. 3 (2) p. 125 and described on p. 126.

If the result is desired in terms of volumes per cent of O_2 or CO capacity, factor f is taken from the last column of Table II of Van Slyke and Neill (1). If the result is desired in terms of mM of O_2 or CO with which the hemoglobin of a liter of blood can combine, factor f is taken from the last column of their Table III.

The value of c is determined by a blank analysis in which the procedure described above is repeated in every detail, except that 2 cc. of water are substituted for the 2 cc. of blood. The correction c is calculated as

$$c = p_1 - p_2$$

The value of c is so small and insensitive to ordinary variations of laboratory conditions that, once exactly determined, it can be used as a constant. In our laboratory, with a temperature of 20–25° and barometric pressure of 740 to 780 mm., the value of c is 4.0 mm., of which 1.4 mm. are due to air admitted in the ferricyanide and 2.6 mm. to CO physically dissolved by the equilibrated blood solution (see Table III).

The cleaning of the chamber, after each analysis, is performed by rinsing with three successive portions of water, as described on p. 534 of Van Slyke and Neill's paper. To the first water portion a few drops of the 5 N NaOH are added to dissolve particles of methemoglobin adherent to the walls of the chamber. These particles usually form a narrow ring in the upper portion of the broad part of the chamber. They are dissolved in a few seconds by the alkaline solution, the chamber being shaken slowly, with enough mercury in it to raise the solution level up to the particles.

Procedure for Analysis of 1 Cc. Blood Samples.

The procedure is the same as when 2 cc. of blood are used, except that 2.5 cc. of water are taken instead of 4.75 cc., 0.13 cc. of the acid ferricyanide solution is used to liberate the CO, and 0.5 cc. of air-free 1 N sodium hydroxide is used to absorb the CO_2. The amount of CO added is the same, 2 cc. measured at atmospheric pressure. The c correction must be determined with these amounts of reagents. The calculation is the same, except that the factors in Tables II and III of Van Slyke and Neill (1) are found in the seventh column of each table, for the conditions that

the sample is 1 cc., S is 3.5 cc., and a is 2.0 cc., or the sixth column, when the CO pressure is determined with the gas at 0.5 cc. volume. With blood of less than ordinary hemoglobin content, more exact results will be obtained by using the 0.5 cc. a mark of the extraction chamber instead of the 2.0 cc. mark for the gas reading.

Procedure for Analysis of 0.1 or 0.2 Cc. Blood Samples.

The blood is drawn into a capillary pipette calibrated to *contain* either 0.1 or preferably 0.2 cc. (It should hold, when filled to the mark with dry mercury, 1.355 or 2.71 gm. of Hg.)

If the blood sample is drawn outside the laboratory, the pipette is delivered into a small test-tube, of about 1 or 2 cc. capacity, containing 0.5 cc. of water for a 0.1 cc. blood sample, 1.0 cc. of water for a 0.2 cc. sample. The pipette is rinsed twice by drawing up into it the supernatant water. The blood and water in the tube are then mixed with a fine rod or wire.

Before the sample thus diluted is transferred to the Van Slyke-Neill apparatus a drop of caprylic alcohol is drawn into the capillary below the cup of that apparatus. The blood solution from the test-tube is transferred to the cup, and thence, with the drop of caprylic alcohol, down into the chamber. The test-tube and cup are washed with repeated water portions of 4 or 5 drops each, until the volume of solution inside the chamber extends exactly to the 2 cc. mark.

If the blood is drawn in the laboratory, the pipette may be rinsed directly into 1 cc. of water previously placed in the cup of the gas apparatus. The blood is mixed with the water by stirring or shaking, and is delivered, together with a drop of caprylic alcohol, into the chamber. The cup is then washed with repeated small portions of water until the solution in the chamber reaches to the 2 cc. mark.

In the same manner described above for analysis of 2 cc. blood samples, 2 cc. of CO gas are added and equilibrated with the blood solution.

Of the ferricyanide solution only 0.05 cc. is added. It is run in while the blood solution is still in the top of the chamber, and is followed by several drops of mercury, which break up the methemoglobin precipitate into fine particles.

The chamber is evacuated and shaken 3 minutes. The fluid is permitted to rise as near to the cock at the top of the chamber as it will with the leveling bulb at the mid-position. (See third paragraph, p. 546 of Van Slyke and Neill (1).) 3 or 4 drops of 5 N sodium hydroxide are run into the chamber, followed by a few drops of mercury. The alkali absorbs the CO_2 gas, and, mixed by the falling mercury drops with the dilute blood solution, dissolves the suspended methemoglobin particles. The aqueous meniscus is lowered below the 0.5 cc. mark, and then brought slowly up to it.

After the p_1 reading is taken, the gas is ejected,[2] the cock is sealed with mercury, the meniscus is again lowered to the 0.5 cc. mark, and p_2 is read on the manometer.

The correction, c, is determined by repeating the analysis without addition of blood.

For the *calculation* the factors in the fifth column of Table II or III of Van Slyke and Neill (1) are used, when 0.2 cc. of blood is employed. When the sample is only 0.1 cc. of blood, these factors are multiplied by 2.

EXPERIMENTAL.

Amount of Acid Ferricyanide Solution Required.—In order to minimize the correction for air introduced with the ferricyanide solution, and still avoid the necessity of preliminary extraction of its dissolved air, we have employed a minimum volume of a concentrated solution. The amount of ferricyanide required under the conditions of analysis was ascertained by preliminary experiments, in which 2 cc. portions of blood were treated as above described, except for variations in the amount of ferricyanide and lactic acid added. An example of the results is given in Table I. It is evident that a maximum yield of CO is obtained in Sample 3. As pointed out by Van Slyke and Neill (1), the equivalent of about 1 cc. of 0.1 N acid per cc. of blood must be added with the ferricyanide, to diminish the affinity of Hb for CO, or the CO will not be completely freed.

Tension of CO Required for Quantitative Saturation of Hemoglobin.

With 2 cc. samples of blood plus the usual 4.75 cc. of water in the chamber, varying tensions of CO gas were measured by the

following technique. The mercury in the chamber was lowered to the 50 cc. mark. Carbon monoxide from the Hempel pipette

TABLE I.

Amount of Ferricyanide Necessary to Liberate CO from 2 Cc. of Blood.

Oxygen capacity of blood by Van Slyke-Neill method = 23.27 volumes per cent.

Sample No.	Reagent used.				Volume of reagent added.	CO capacity found.
	Potassium ferricyanide.			Lactic acid, concentrated.		
	Amount used per analysis.	Concentration of solution used.	Volume.			
	mg.	*gm. per 100 cc.*	*parts by volume*	*parts by volume*	*cc.*	*vol. per cent*
1	38.4	8	96	4	0.50	22.83
2	46.0	20	92	8	0.25	22.92
3	76.75	32	92	8	0.25	23.29
4	153.5	32	96	4	0.50	23.15
5	153.5	32	92	8	0.50	23.20

TABLE II.

Tension of CO Required to Saturate Hemoglobin of Ox Blood, as Diluted for Analysis.

Blood No.	O_2 capacity by Van Slyke-Neill method.	Tension of CO at beginning of saturation.	Estimated volume of CO added, measured at 760 mm. and 22°.	Estimated tension of CO at end of saturation.	CO capacity found.	
	vol. per cent	*mm.*	*cc.*	*mm.*	*vol. per cent*	*per cent of O_2 capacity*
1	20.96	15	0.85	7	20.39	97.3
		30	1.70	22	20.97	100.0
		60	3.40	52	21.04	100.4
2	20.88	30	1.70	22	20.82	99.7
3	21.68	30	1.70	22	21.73	100.2
4	21.80	18.6	1.0	10	21.23	97.5
		35.3	2.0	27	21.73	99.8
		70.6	4.0	53	21.76	99.9

(Fig. 1) was then admitted until sufficient had entered to increase the pressure, read on the manometer, by the amounts indicated in Table II as "Tension of CO at beginning of saturation," the mer-

cury level in the chamber still remaining at the 50 cc. level. In fact, as the CO was admitted, the mercury in the chamber did not remain at this level, but fell as part of the mercury passed over into the rising column in the manometer. To bring the mercury back to the 50 cc. mark in the chamber, a little had to be admitted from the leveling bulb, with a consequent further rise also in the manometer column. We found that when sufficient gas was admitted to make the preliminary rise 70 per cent of the CO pressure desired, the above mentioned subsequent admission of mercury from the leveling bulb raised it to the desired pressure. The initial tension of CO could thus be regulated readily within 1 mm. The corresponding volumes of CO in cc. measured under atmospheric conditions are calculated in the fourth column of Table II, for comparison with the 2 cc. volume directed for routine use.

TABLE III.

Determinations of CO and Air in Blank Corrections of Analysis of 2 Cc. of Blood.

Pressures at 2.0 cc. volume.

Sample No.	$P_{CO + air}$ (a)	P_{air} (b)	P_{CO} (a) − (b)
	mm.	*mm.*	*mm.*
1	4.1	1.4	
2	3.9	1.4	
Mean.	4.0	1.4	2.6

The final CO tension was calculated by estimating the amount of CO left free in the chamber after the blood had absorbed the volume that was recovered in the subsequent analysis.

The results in Table II indicate that a final tension over 20 mm. is sufficient to insure complete saturation of the hemoglobin. At a final tension of 7 mm. 97.3 per cent was found saturated.

Relative Amounts of CO and Air in Blank Corrections.

Blank analyses were run as described for 2 cc. of blood. In each analysis 6.75 cc. of water were equilibrated in the gas chamber with 2 cc. of carbon monoxide gas. Then the free gases were ejected, 0.25 cc. of acid ferricyanide was added, the gases were extracted, and the pressure $P_{co + air}$ was measured at 2 cc. volume.

For P_{air} the same procedure was used, except that no CO was admitted. The gases measured by the pressure P_{air} after the second extraction in this case were derived from the air introduced with the 0.25 cc. of ferricyanide, plus a negligible trace of air not removed by the first extraction. The results are given in Table III.

Table IV indicates the order of constancy of the results obtained by the oxygen capacity method of Van Slyke and Neill (1) and by the present carbon monoxide capacity method applied with blood

TABLE IV.

Macro and Micro Determinations of CO Capacity of Blood Compared with O_2 Capacities by Van Slyke-Neill Method.

Blood No.	O_2 capacity.	CO capacity.			
	2 cc. samples.	2 cc. samples.	1 cc. samples.	0.2 cc. samples.	0.1 cc. samples.
	vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*
1 a	18.96	18.83	18.84	18.82	18.20
b	19.08	18.92	19.02	18.80	18.80
c	18.97	18.87	19.05	18.88	18.67
Average.	19.00	18.87	18.97	18.8	18.6
2 a	21.25	21.36	21.47	21.29	21.13
b	21.26	21.35	21.43	21.26	22.06
c	21.31	21.40	21.47	21.34	21.27
Average.	21.27	21.37	21.46	21.3	21.5
3a	22.35	22.52	22.50	22.29	22.70
b	22.45	22.42	22.38	22.51	22.31
c	22.37	22.42	22.29	22.50	22.20
Average.	22.39	22.45	22.39	22.4	22.4

samples of 2, 1, 0.2, and 0.1 cc. With blood samples down to and including 0.2 cc. the gasometric CO method, if carried out in detail according to directions, is probably more exact than any colorimetric method. When the sample is reduced to 0.1 cc., however, variations of as much as ±2 per cent of the amount measured may occur in the gasometric procedure. In this case it has over colorimetric methods only the advantage of freedom from errors due to inaccurate or unstable colorimetric standards.

SUMMARY.

Hemoglobin is determined by measurement of its capacity to combine with carbon monoxide. The whole process of saturation of the blood with carbon monoxide and its subsequent analysis is carried out in the manometric gas apparatus of Van Slyke and Neill. Amounts of blood varying from 2 cc. to 0.1 cc. can be used.

The agreement of carbon monoxide capacities obtained by this method with oxygen capacities determined by the technique previously employed in this laboratory (1, 3, 4) confirms, with perhaps more exactness than previously available data, the fact that the hemoglobin of blood combines with identical volumes of oxygen and carbon monoxide.

BIBLIOGRAPHY.

1. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, lxi, 523, (554).
2. Van Slyke, D. D., *J. Biol. Chem.*, 1927, lxxiii, 121.
3. Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.
4. Van Slyke, D. D., and Stadie, W. C., *J. Biol. Chem.*, 1921, xlix, 1.
5. Van Slyke, D. D., *J. Biol. Chem.*, 1927, lxxiii, 698.
6. Van Slyke, D. D., *J. Biol. Chem.*, 1926–27, lxxi, 235.

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PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

Twenty-Second Annual Meeting.

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QUANTITATIVE RELATIONS OF THE LIPIDS OF NORMAL TISSUES.

By W. R. BLOOR.

(*From the Department of Biochemistry, the University of Rochester School of Medicine and Dentistry, Rochester, New York.*)

This is a report of a study of the nature and quantitative relationships of the lipids of normal tissues of cattle, constituting the first part of an investigation into the functions of lipids in cell life. The lipids were extracted quantitatively, separated into fractions, and the fractions examined as to their content of fatty acids and unsaponifiable matter, also as to the nature of the fatty acids found. It was found (1) that each tissue had a fairly constant content of phospholipid and unsaponifiable substance which was to a considerable extent characteristic of the tissue although quite wide limits of variation were found; (2) that the content of fat in each tissue was not constant or characteristic although the tissues could to a certain extent be grouped according to their fat content; (3) that the nature of the lipids in different tissues was not greatly different although small and apparently characteristic differences, in particular, in the degree of unsaturation of the fatty acids, were noted; (4) that the outstanding difference between the lipids of various tissues thus appeared to be in their percentage amounts rather than in their nature.

THE NEW SULFUR-CONTAINING AMINO ACID DESCRIBED BY J. H. MUELLER.

By GEORGE BARGER and F. P. COYNE.

(*From the Department of Medical Chemistry, University of Edinburgh, Edinburgh, Scotland.*)

A few years ago J. Howard Mueller[1] found a new sulfur-containing amino acid in casein and showed[2] that its neutral sulfur

[1] Mueller, J. H., *J. Biol. Chem.*, 1923, lvi, 157.
[2] Mueller, J. H., *J. Biol. Chem.*, 1923–24, lviii, 373.

is oxidized in the organism to an inorganic form. Soon afterwards Odake[3] obtained a minute quantity of the same substance from yeast. The constitution $CH_3 \cdot S \cdot CH_2 \cdot CH_2 \cdot CH(NH_2)COOH$, already suspected by Dr. Mueller, is now suggested for it, on account of the almost certain identity with α-amino-γ-methylthiol-*n*-butyric acid, synthesized for the purpose by the Strecker method. Accordingly the name *methionine* is proposed for it, after consultation with Dr. Mueller, whose generous help is hereby gratefully acknowledged. Methionine is evidently the parent substance of cheirolin (from the seeds of the wallflower *Cheiranthus cheiri*), studied and synthesized by Schneider;[4] this substance has the constitution $CH_3 \cdot SO_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot N{:}C{:}S$ and is thus the methyl sulfone of propylthiocarbimide.

THE EFFECT OF THE ADMINISTRATION OF CREATINE ON THE BLOOD SUGAR.

By ROBERT M. HILL.

(From the Laboratory of Biochemistry, School of Medicine, University of Colorado, Denver.)

Creatine (30 mg. per kilo) was administered subcutaneously to eight dogs. There was a drop in the blood sugar of from 16 to 24 mg. per 100 cc. The drop was at a maximum at the 1st or 2nd hour and lasted more than 4 hours. When 100 mg. of creatine per kilo were given by mouth the drop in the blood sugar was about the same and lasted somewhat longer. Increasing the amount of creatine given by mouth to 400 mg. per kilo did not cause any greater decrease in the blood sugar. In an experiment in which glucose was fed, followed 2 days later by the same feeding plus 100 mg. of creatine per kilo, the rise in the blood sugar curve, in the second case, was from 30 to 50 mg. per kilo less, and the fall was more rapid, reaching values 10 to 20 mg. below the value before the experiment started. Two similar experiments with normal human subjects in which 50 mg. of creatine per kilo were fed gave like results. In one case there was a fall in the blood sugar of 25 mg. per 100 cc. without any rise, after 1.75 gm. of glucose and 50 mg. of creatine per kilo were fed.

[3] Odake, S., *Biochem. Z.*, 1925, clxi, 446.

[4] Schneider, W., *Ann. Chem.*, 1910, ccclxxv, 207.

A REDUCING URINARY TYROSINE COMPOUND IN CASES OF A MUSCULAR DISEASE (MYASTHENIA GRAVIS).

By HILDING BERGLUND, GRACE MEDES, and ANNE LOHMANN.

(*From the Department of Medicine, University of Minnesota, Minneapolis.*)

In the course of the study of the urine from a patient with myasthenia gravis a compound was discovered which readily reduces phosphomolybdic acid both in acid and in alkaline solution, phosphotungstic acid in alkaline solution, as well as alkaline copper solution. The readiness with which the phosphomolybdic acid is reduced is in contrast to the slowness of the reduction of the copper, which is reduced decidedly more slowly by this compound than by glucose. It was readily differentiated from homogentisic acid.[5]

The compound has been isolated from the urine of the patient by the following procedure: evaporation of the urine on a water bath; extraction of the residue with methyl alcohol (about 85 per cent); evaporation of the extract under reduced pressure; dissolving in absolute methyl alcohol; subsequent precipitation with ether. Further purification is obtained by alternately dissolving in absolute methyl alcohol and precipitating with ether ten to sixteen times.

In the first stages of purification the precipitate comes down in gummy masses until it has been fully freed from water, a process which requires about three precipitations. Subsequently a fine reddish flocculent precipitate is obtained, which with increasing purification comes down with greater difficulty. The precipitate is each time separated by centrifugalization. The supernatant liquid, which at first is brownish, later on becomes pale yellow. The precipitate is finally washed several times with ether to remove all traces of methyl alcohol and dried with slight heat in a stream of dry air. The dried fine precipitate has a pale yellow color.

An aqueous solution of the purified compound, when precipitated with phosphomolybdic acid, gives a voluminous, dark blue precipitate with a greenish supernatant liquid. This is considered

[5] In two other milder cases of myasthenia gravis a similar though slight reduction occurred. It was not observed in other cases of muscular diseases characterized by creatinuria, nor in urines from other patients or normal individuals.

proof that the purified compound is identical with the compound which, through its reduction of the phosphomolybdic acid in the course of phosphate determinations, first drew our attention to the peculiar behavior of the urine excreted by our patient with myasthenia gravis.

The purified compound, when determined by the Folin and Ciocalteu method for the determination of tyrosine, gives a blue color which exactly matches the color developed by the tyrosine standard, and has a reducing value one-fifth as great as the latter.

The purified compound is highly soluble in water, somewhat less so in absolute methyl alcohol, almost insoluble in cold absolute ethyl alcohol, and totally insoluble in ether and benzene. It gives a strong Millon's reaction, and negative biuret, ninhydrin, and Hopkins-Cole reactions. Schiff's reaction is negative. In aqueous solution it may be precipitated with neutral lead acetate, silver nitrate, and mercuric chloride.

The compound contains both nitrogen and phosphorus, the former increasing and the latter decreasing during the progress of purification, until constant figures are obtained; N, 7 per cent; P, 3.1 per cent. There is no inorganic phosphorus present. The complete oxidation of the phosphorus is difficult to accomplish. The fixed base is 4.6 per cent. On the basis of 1 atom of phosphorus, this would indicate a molecular weight of 1000, with 5 nitrogen atoms and 2 sodium atoms.

As already indicated a strong Millon's reaction is obtained. Observations on the patient have shown that the feeding of tyrosine in doses of 2.5 and 5 gm. a day produces a definite increase in the excretion of the compound, while such an increase is absent when glycine is fed in the same doses. Feeding and injection experiments with the purified compound are under way. Further chemical studies of the compound are likewise in progress.

EXPERIMENTAL STUDY OF PLASMA PROTEIN REGENERATION.

By FREDERIC W. SCHLUTZ, W. W. SWANSON, AND MILDRED R. ZIEGLER.

(From the Department of Pediatrics, University of Minnesota, Minneapolis.

It has been shown by Kerr and his associates[6] and by Smith and his associates[7] that hemorrhage is followed by a gradual restoration of the plasma protein fractions. The one most rapidly restored appears to be fibrinogen, followed by the globulins, and finally the albumins. It was the object of this study to investigate the regeneration of the several fractions into which the globulins and albumins may be divided. No general anesthetic was given the animals (dogs) used in these experiments.

Young dogs were employed for all the experiments. They were fed a modification of Cowgill's low protein diet for dogs.[8] The animals ate well and maintained their original weight until bled. A few lost considerably after bleeding.

The dog under observation was suspended from the ceiling in a canvas hammock, a device used in Mendel's laboratory for administering a stomach tube. The hind leg, just below the knee joint, was shaved and anesthetized with a 2 per cent procaine solution. A large vein was exposed and a cannula inserted. By successive bleedings, between 60 and 75 per cent of the dog's original blood volume was taken.

The total protein nitrogen was determined by the macro-Kjeldahl method, the non-protein nitrogen by the technique of Folin. The fractionation of the proteins into fibrin, euglobulin, pseudoglobulin, and the three albumin fractions was carried out according to an unpublished method of Berglund. The nitrogen in each fraction was determined by a macro-Kjeldahl digestion and titration with 0.5 N alkali.

A review of the tables of five dogs shows the following results. The plasma protein, except in one dog (No. 4), dropped after the bleeding from 20 to 40 per cent. Care was taken that the cell

[6] Kerr, W. J., Hurwitz, S. H., and Whipple, G. H., *Am. J. Physiol.*, 1918, xlvii, 356, 370, 379.

[7] Smith, H. P., Belt, A. E., and Whipple, G. H., *Am. J. Physiol.*, 1920, lii, 54.

[8] Cowgill, G. R., *J. Biol. Chem.*, 1923, lvi, 725.

volume did not fall below 20 per cent by volume. The fibrinogen uniformly showed increase.

On the whole, the increase in euglobulin parallels the increase in fibrinogen. The pseudoglobulin seemed to be influenced most by the bleeding. In every instance, it was reduced to approximately below 50 per cent of the original amount. Restoration took place slowly and was not as completely accomplished as in the other two globulin fractions.

Albumin Fraction I in practically all of our experiments shows a definite increase over the original amounts present in the plasma. Albumin Fraction II, which originally is present in only small amount, shows some tendency to restoration but, on the whole, is the least responsive fraction to bleeding. Albumin Fraction III is most influenced by the bleeding and its restoration delayed longer than in any of the other fractions.

The total non-protein nitrogen showed a definite increase in all of the experiments. Comparing the total restoration of the globulins with the restoration of albumin and expressing it in terms of albumin-globulin ratio, one could say from the figures that there seems to be a definite shift in the values toward the globulins as the regeneration takes place.

These findings would seem to be in accord with a theory expressed by Herzfeld and Klinger[9] who believe that protein fractions do not have a chemical individuality but are interrelated series of colloidal particles of different degree of dispersion, which can be transposed one into the other. The evolution begins with the lowest dispersed particles, fibrinogen, and extends to the albumins and non-coagulable substances.

FURTHER STUDIES ON AMMONIA FORMATION.

By SIDNEY BLISS.

(From the Department of Biochemistry, McGill University, Montreal, Canada.)

A study has been made of the possible existence in blood of complex ammonia compounds; *i.e.*, ammonia which is not yielded by aeration from an alkaline medium.

[9] Herzfeld E., and Klinger, R., *Biochem. Z.*, 1917, lxxxiii, 228; 1919, xcix, 204; *Ergebn. Hyg., Bakt., Immunitätsf. exp. Therap.*, 1920, iv, 282.

On the assumption that ammonia, formed in tissues, is transported in non-toxic form, combined with some other blood constituent, it might be expected that the kidney possesses an enzyme capable of liberating ammonia from such a linkage. Such an enzyme was found in kidney tissue.

With the use of this enzyme it is found that the "ammonia" content of blood is at least a thousand times the present low figure for blood.

The high absolute value for the "ammonia" of blood dispenses entirely with the necessity for assuming that the kidney plays a special rôle in ammonia formation, and the original statement of the author that ammonia formation is a general tissue phenomenon is confirmed with the new high values. The present values also explain the large quantities of vomitus ammonia previously found in nephritics.

The fact that renal venous blood gave higher ammonia value than arterial blood by the older method is probably due to the fact that renal venous blood may contain traces of the kidney enzyme that yield small amounts of ammonia before the estimation can be completed, and are hence entirely accidental in character.

The particular feature of the new method lies in the fact that the absolute values fluctuate with the state of acid-base balance.

The combined ammonia in blood is to be carefully distinguished from the idea of an ammonia precursor or ammonia mother substance. It is shown that the ammonia is formed in the tissues, and the form present in blood is derived from metabolic ammonia; metabolic ammonia is not formed from it.

In contrast with the present values of 0.05 to 0.10 mg. of ammonia nitrogen per 100 cc. of blood, the new values are 118 to 125 mg. of nitrogen per 100 cc. of blood in both the cubital vein of man and the femoral artery of the dog.

The present high values are shown to be of significance in the acid-base economy of the body by reason of fluctuations that occur when the acid-base balance is disturbed.

It is suggested that the "ammonia" of blood exists there in the form of amide nitrogen.

POTASSIUM CONCENTRATION IN MUSCLE CELLS AS INFLUENCED BY THE DONNAN EQUILIBRIUM.

By PHILIP H. MITCHELL.

(From the Biological Laboratory of Brown University, Providence.)

The potassium content of frog muscle when computed as per cent of the muscle solids shows a fairly definite relationship to the water content of the muscle. The water content varies even in frogs kept in an aquarium. The usual limits are 78.5 per cent to 82.5 per cent. Occasionally the water content is higher. It can be made lower, even as low as 72.5 per cent, by depriving the frogs of water. When potassium as per cent of muscle solids is plotted as ordinates against percentage of water content as abscissas, the curve is nearly flat for lower values of the water but rises regularly and steeply with higher water content. The greatest inflection of the curve is between 78.5 and 81 per cent of water. It thus appears that change in the concentration of cell potassium, as a result of water loss or gain, causes outward or inward diffusion of potassium across the cell membrane. Potassium loss, however, slows down upon approaching a certain minimum value, about 1.50 per cent of muscle solids. If well nourished frogs, with food still in the stomach or that were very recently taken from their natural summer habitat are used, a similar curve is obtained, but its potassium values are at higher levels. This indicates that rise in the potassium content of the blood increases the potassium of muscle cells. Perfusion of frog muscle with potassium-free isotonic salt solution causes similar relationships to appear but with lower potassium values so that decrease of potassium concentration of the cell medium causes loss of cell potassium. These observations are interpretable on the assumption that the forces set up by a Donnan equilibrium control the potassium content of cells.

ON THE PRESENCE OF ALUMINUM IN ANIMAL TISSUES.

By VICTOR C. MYERS, J. W. MULL, and D. B. MORRISON.

(From the Departments of Biochemistry, School of Medicine, Western Reserve University, Cleveland, and the State University of Iowa, Iowa City.)

The lake produced by aluminum on the ammonium salt of aurintricarboxylic acid, described by Hammett and Sottery, has been made the basis of a colorimetric method of estimating the very small amounts of aluminum present in animal tissues. With the technique used such interfering elements as iron, magnesium, and calcium are removed, and it is possible to recover aluminum added at various stages in the determination, and to distinguish between amounts varying by 0.003 mg. With this method analyses have been made of tissues of white rats and dogs on different diets and of human autopsy material. In the rat the largest amount of aluminum was found in the liver. In twenty-eight control rats the average aluminum content was 0.130 mg. per 100 gm., in thirty-four rats receiving aluminum, 0.161 mg., and 0.063 mg. in eight rats on a diet as free as possible from aluminum. Following intraperitoneal injection the aluminum content of the liver in two animals averaged 8.20 mg. per 100 gm. Analyses of different tissues were made on seventeen dogs, ten of which were receiving aluminum. In general the figures found were slightly higher than for the rat. Of seven control dogs the average figure for the liver was 0.150 mg. per 100 gm. and 0.266 mg. for seven dogs receiving aluminum. In human autopsy material much higher figures have been found in the brain and heart than in the liver. Analyses from ten autopsies give the following averages calculated as mg. of aluminum per 100 gm.: heart, 0.225 mg.; brain, 0.203 mg.; liver, 0.074 mg.; and gallbladder with bile 0.069 mg.

THE ANALYSIS OF LIVER EXTRACT.

By JOSEPH M. LOONEY.

(From the Department of Physiological Chemistry, Jefferson Medical College, Philadelphia.)

Extracts of potent material were made from fresh liver by a modification of the process outlined by Cohn. The livers were

macerated with 10 times their weight of water made slightly alkaline with NaOH. The mixture was brought to pH 5.0 and then heated to 70° before filtering. The filtrate was concentrated *in vacuo* and made to contain 60 per cent alcohol by the addition of 95 per cent alcohol. The solution was filtered and poured into enough absolute alcohol to make the final strength 90 per cent. The gummy residue was dehydrated with more absolute alcohol and washed with ether.

This material was precipitated by phosphotungstic acid and by picric acid and gave a biuret reaction of a polypeptide. It contained 10.6 per cent N, 0.47 per cent P, 3.33 per cent tyrosine, 2.38 per cent cystine, and no tryptophane.

The fraction from the 60 per cent alcohol was not completely soluble in water and contained 8.0 to 9.0 per cent tyrosine. The fraction 60 to 90 per cent was readily soluble in water and appeared to contain most of the active principle.

The material was proved to be beneficial in most cases; only one case out of a series of eight was not helped.

THE YELLOW PIGMENT OF SERUM AND MUSCLE.

By DAVID L. DRABKIN.

(*From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia.*)

The yellow coloring matter of the aqueous extract of voluntary muscle and of blood serum has been extracted by means of normal butyl alcohol.

An aqueous extract of dog muscle was obtained by boiling ground fresh muscle in water. The filtered extract was acid (pH about 5.7) and was yellow in color. After acidification to pH about 4.0 most of the pigment was extracted from the aqueous solution by shaking with butyl alcohol. The color of the butyl alcohol solution, after concentration *in vacuo*, was red. This pigment was extracted under identical conditions used for obtaining the normal urinary pigment.[10] A preliminary study of the muscle pigment has indicated that it may prove to be related chemically to the pigment of the urine. A solution of the muscle

[10] Drabkin, D. L., *J. Biol. Chem.*, 1927, lxxiv, p. xv.

pigment gives a positive test for tyrosine, has reducing properties, *etc.* This pigment has not been studied hitherto.

The yellow pigment of dog serum could not be extracted directly by use of normal butyl alcohol. The serum, diluted with an equal volume of distilled water, was made slightly acid to litmus and was heated to boiling. The colorless coagulated protein was filtered from the pale yellow aqueous solution. On further acidification of the filtrate, the pigment was completely removed from it by a single extraction with butyl alcohol. The butyl alcohol solution was concentrated *in vacuo*. The orange-red color of this concentrate has not faded after several months. The reactions of the solution and its qualitative spectrum show no resemblance to solutions of carotin. It has been found, however, that the serum pigment differs from the normal urinary pigment in that it does not, with increase in alkalinity, pass from butyl alcohol into water.[10]

Highly colored horse serum was treated in the same way as the serum of the dog. In this case, the coagulated protein was highly colored. The yellow pigment of the filtrate was similar to that obtained from dog serum in that it resembled neither carotin nor the urinary pigment. The pigment which separated with the coagulated protein could be removed by the method of Palmer[11] or by extraction with butyl alcohol. The solution obtained possessed the chemical and spectroscopic characteristics of carotin solutions. On standing it became almost colorless. It is of interest to note also the finding that butyl alcohol readily extracted the carotin from untreated horse serum.

A FURTHER STUDY OF MENSTRUAL VARIATIONS IN BLOOD COMPOSITION IN NORMAL WOMEN.

By RUTH OKEY.

(*From the Laboratory of Household Science, University of California, Berkeley.*)

This series of studies was undertaken in order to ascertain whether or not the menstrual symptoms varying from slight discomfort to actual pain and nausea so often observed in otherwise normal women were consistently associated with any variation in

[11] Palmer, L. S., Carotinoids and related pigments, New York, 1922, 208.

blood composition. Observations, unless otherwise indicated, were made on blood samples taken 14 hours after the last meal and at 2 to 3 day intervals throughout a period of at least 1 month for each subject studied. *Alkali reserve* as indicated by the determination of CO_2-carrying capacity by the Van Slyke method in nine women seems to be rather consistently lowered from 5 to 8 volumes per cent just before the onset of menstruation. *Chlorides* as estimated by the Whitehorn method show comparatively little day to day variation, but values for the day of onset of menstruation were, in nine cases out of twelve, 10 to 40 mg. higher than the general average. *Inorganic phosphate* as estimated by the method of Fiske and Subbarow likewise shows a rather small day to day variation, but values 0.2 to 0.7 mg. higher than the average seem to be the rule at the time of onset of menstruation. Our studies to date have indicated that the *calcium* level is apparently more variable in women than in men. There seems moreover to be a tendency to a fall in calcium level associated with the cramp-like pain and nausea which sometimes accompany the onset of menstruation. The women studied have, during the intermenstrual period, responded to the ingestion of 10 gm. of calcium lactate with an average maximal rise in blood calcium amounting to about 12 to 14 per cent of the fasting level. Nevertheless, a number of the subjects who have shown this reaction intermenstrually have failed to show any increase in blood calcium level following calcium ingestion during the first few hours of the menstrual period; *i.e.*, the time most frequently characterized by cramp-like pain. Because most of our calcium determinations have been made on plasma by the G. W. Clark method and some of our figures are so low as to raise the question of analytical error, we are now repeating the calcium study using the Clark-Collip method with serum.

In general our studies would suggest that the endocrine disturbance which is associated with menstruation may manifest itself in characteristic changes in the relative concentration of certain inorganic blood constituents.

STUDIES ON GUANIDINE COMPOUNDS.

THE EXCRETION OF GUANIDINE BASES AFTER SUBCUTANEOUS OR ORAL ADMINISTRATION.

By CLARENCE J. WEBER.

(From the Department of Experimental Medicine, University of Kansas School of Medicine, Lawrence.)

The determination of the guanidine bases in urine is made by adsorption with norit and subsequent release and colorimetric determination with a ferricyanide-nitroprusside reagent. Although the reagent is not absolutely specific for these bases it has proved extremely useful for the type of work for which we have used it.

Several investigators have endeavored to show a causal relationship between certain pathological conditions and the guanidine bases. We believe that a knowledge of the fate of these bases when administered to the normal animal would permit a better evaluation of the results obtained in pathological conditions.

Guanidine, methylguanidine, and *as*-dimethylguanidine were administered orally and injected subcutaneously in toxic and nontoxic amounts into rabbits and dogs. Our results show rapid elimination of these compounds in the urine, 90 to 95 per cent of the administered base being excreted. The guanidine group of these compounds is not destroyed, nor is there evidence of any other type of detoxication for these compounds after subcutaneous or oral administration.

THE CYSTINE CONTENT OF PHASEOLIN.

By M. X. SULLIVAN.

(From the Division of Chemistry, the Hygienic Laboratory, United States Public Health Service, Washington.)

Phaseolin, the β-globulin of the navy bean (*Phaseolus vulgaris*) has been extensively studied both from the view-point of its constituent amino acids and from its biological value in feeding experiments. Chemical tests have indicated the presence of all the known essential amino acids, whereas feeding experiments have shown the need of adding cystine to make a diet containing phaseo-

lin as the sole protein satisfactory for the well being of rats. As found in the literature cystine determinations by the Van Slyke procedure and by the Folin-Looney method have indicated a sufficient supply of cystine. Work with the Sullivan method of determining cystine in the hydrolysates of foodstuffs[12] has shown little if any cystine in phaseolin,—a finding in keeping with the results of feeding experiments.

THE ACTION OF ACIDS IN PRODUCING HEMOLYSIS.

By MEYER BODANSKY.

(From the Laboratory of Biological Chemistry, School of Medicine, University of Texas, Galveston.)

The effective range of hydrogen ion concentration required to produce rapid hemolysis has been determined for hydrochloric, nitric, sulfuric, and phosphoric acids, and for the members of the saturated fatty acid series from formic to capric. At a given hydrogen ion concentration the inorganic acids are much less hemolytic than the fatty acids. The effectiveness of a fatty acid in producing hemolysis is related to the size of the non-polar portion of its molecule and to the reciprocal of its water-oil distribution coefficient.

EXPERIENCES WITH THE PHOSPHATASE DESCRIBED BY ROBISON.

By R. J. TITHERINGTON and WITHROW MORSE.

(From the Department of Physiological Chemistry, Jefferson Medical College, Philadelphia.)

The enzyme was obtained from various sources—rabbit, guinea pig, and calf, and from different organs, bone, liver, and kidney. In general the reports of Robison and collaborators were confirmed. The curve of hydrolysis varies accordingly as the digest is buffered or not. By precipitating the proteins of the enzyme substance by means of $(NH_4)_2SO_4$ and alcohol, a solution was obtained which still gave the typical biuret reaction for true proteins but fainter. Such a preparation of enzyme showed, still, 42 per cent hydrolysis in 4 hours, as compared to a hydrolysis of 75.4 per cent for

[12] Sullivan, M. X., *J. Biol. Chem.*, 1927, lxxiv, p. xiv.

enzyme obtained by the Robison scheme. The enzyme seems to have an optimum temperature of 55–58°. At 60°, the rate of hydrolysis is 15.4 per cent. The enzyme was found to be active after 7 days in the dead body of a guinea pig, with a hydrolysis rate of 29.1 per cent in 4 hours. The following rates of hydrolysis were determined (4 hours hydrolysis):

	per cent
Rabbit, 60 per cent grown, bone	55.1
Guinea pig, 75 per cent grown, bone	40.2
Calf, bone	75.4
" kidney	66.2
" liver	45.0

THE ACTION OF ULTRA-VIOLET RAYS ON VITAMIN B.

By ALBERT G. HOGAN and JESSE E. HUNTER.

(From the Departments of Animal Husbandry and Agricultural Chemistry, College of Agriculture, University of Missouri, Columbia.)

We exposed carriers of vitamin B, in the form of a dry powder, to a quartz mercury arc for a period of 10 hours, and observed that their vitamin activity was greatly reduced. Such preparations were still effective in curing pigeons of polyneuritis, but they would not sustain life, either of pigeons or of rats, for more than a few weeks. Apparently the antineuritic activity of vitamin B is unimpaired by exposure to ultra-violet rays, but some other essential property is destroyed. It is well established that autoclaved vitamin B loses its antineuritic potency, but that it retains activity of some other kind, usually described as "growth-promoting."

The facts outlined above suggest the possibility that vitamin B is a mixture of two components, and that one is destroyed by irradiation, the other by high temperatures. If our hypothesis is correct, there should be a supplementary relation between the two preparations, the one obtained by heating to a high temperature, the other by exposure to a quartz mercury arc, and we believe this is the case.

Rats and pigeons were used as experimental animals, and both yeast and a yeast concentrate were used as a source of vitamin B. In each series of observations the animals were divided into four

groups corresponding to the four modifications of the vitamin that were used. Group 1 received the irradiated supplement, and Group 2 received the autoclaved material. Each member of the third group received one-half as much of the irradiated supplement as did the individuals of Group 1, and in addition one-half as much of the autoclaved supplement as did the individuals of Group 2. The fourth group received untreated material. Our chief criteria in judging the adequacy of the various supplements were the survival periods both of the pigeons and rats, and the rate of growth of rats. There were no survivors in either Group 1 or Group 2. Groups 3 and 4 were indistinguishable, among both rats and pigeons. We conclude, therefore, that vitamin B is a mixture.

VITAMIN REQUIREMENTS OF NURSING YOUNG.

IV. (a) A QUANTITATIVE BIOLOGICAL METHOD FOR THE STUDY OF VITAMIN B REQUIREMENTS OF NURSING YOUNG. (b) MARKED ANHYDREMIA ASSOCIATED WITH MARKED DISTURBANCE IN HEMATOPOIETIC FUNCTION OF NURSING YOUNG SUFFERING FROM VITAMIN B DEFICIENCY.

By BARNETT SURE, M. C. KIK, AND DOROTHY J. WALKER.

(*From the Laboratory of Agricultural Chemistry, Arkansas Agricultural College, Fayetteville.*)

(*a*) The quantitative method for the study of vitamin B requirements of nursing young is essentially an elaboration of the recent method for the study of vitamin B requirements for lactation.[13] Instead of depleting the lactating mothers of all vitamin reserves, the preventive method is employed by administering daily small amounts of highly concentrated vitamin B preparations to the mother from the date of birth of the litter. Vitamin therapy to the nursing young is begun as early as the 7th day of lactation, by use of graduated minimum increasing dosages of the same vitamin concentrate given the mother, the daily dosages being determined according to needs of the litter. With such technique dozens of litters were successfully weaned on a minimum daily dosage of 7 to 12 mg. per nursling, the lactating mother receiving a daily allowance of only 5 to 10 mg.

[13] Sure, B., *J. Biol. Chem.*, 1928, lxxvi, 673.

(*b*) *Marked Anhydremia.*—In 56 nursing young in the state of incipient polyneuritis and prolonged maintenance an increase in the concentration of the blood of 23.4 to 43.8 per cent was obtained. The percentage of total blood solids was compared with forty-four normal animals of the same age.

Marked Disturbance in Hematopoietic Function.—For this study we have completed work to date on 444 animals, 114 pathological, and 330 normals, using animals of the same age. Hemoglobin determinations (Newcomber method) and erythrocyte counts, while decidedly higher in the pathological animals than in the controls, show considerable disturbance in hematopoietic function unaccounted for by the severe anhydremia. Most animals show a color index above 1.0 and cases of 1.5 to 2.0 were frequently encountered.

COMPARISON OF THE ANTIRACHITIC POTENCY OF ERGOSTEROL IRRADIATED BY ULTRA-VIOLET LIGHT AND BY EXPOSURE TO CATHODE RAYS.

By ARTHUR KNUDSON and CHESTER N. MOORE.

(*From the Laboratory of Biological Chemistry, Medical Department of Union University, Albany Medical College, and Research Laboratory, General Electric Company, Albany.*)

Reports from these laboratories have shown that antirachitic properties can be induced in various substances, such as cholesterol, yeast, cottonseed oil, *etc.*, by exposure to cathode rays. It is now well recognized that the sterol ergosterol is converted by the action of ultra-violet light into a very potent antirachitic substance so that as little as 0.0001 mg. of irradiated ergosterol per day brings about healing of rickets in a rat. Experiments on the antirachitic activity of ergosterol produced by exposure to cathode rays were therefore undertaken in order to determine the best procedure for obtaining the most potent product and to compare this potency with that obtained by ultra-violet radiation.

In one group of experiments with cathode rays ergosterol has been exposed in a dry form in air at room temperature and at the temperature of liquid air. It has also been exposed in an atmosphere of nitrogen in dry form and dissolved in alcohol. The exposure in all these cases was 30 seconds, at an average distance

of 2 inches from the window and with a current of 1 milliampere and 180,000 to 200,000 volts. Under these various conditions it was found that the antirachitic potency was about the same and that 0.0025 mg. was the lowest dose which brought about marked healing of rickets in a rat. In one experiment the ergosterol was dissolved in alcohol and the cathode ray exposure was continued for 5 minutes in an atmosphere of nitrogen, and as much as 0.0125 mg. was necessary to bring about distinct healing of rickets.

In another group of experiments ergosterol was exposed in a dry form but the time interval was varied. Exposures of 0.2, 1, 5, 30, and 180 seconds were tried and it was found that with an exposure of 5, 30, and 180 seconds about the same potency was obtained. With exposures of 0.2 and 1 second the products were considerably less potent. With a 1 second exposure 0.0125 mg. had to be given daily to show marked healing of rickets.

In most of the experiments with ultra-violet radiation the ergosterol was dissolved in alcohol (1 mg. per 5 cc.) and rayed in a quartz cell. Exposures of 300 minutes, 30 minutes, 3 minutes, 15 seconds, and 1 second were studied. It was found that after irradiation of ergosterol for 30 minutes it was markedly potent in doses of 0.0001 mg. per day. An exposure of 15 seconds gave about the same potency and with 3 minutes irradiation the product was somewhat more potent than with 30 minutes irradiation. An exposure of 1 second required a dose of 0.0005 mg. to bring about marked healing of rickets. With irradiation of ergosterol for 5 hours it was found that 0.0125 mg. was ineffective in bringing about healing of rickets; as though some of the antirachitic activity had been destroyed.

With the cathode ray exposure the lowest dose which would bring about effective healing of rickets was 0.0025 mg. and with ultra-violet radiation as little as 0.0001 mg. brought about a marked healing and even in one or two instances as little as 0.00002 mg. showed indications of healing of rickets in rats. These experiments would indicate therefore that under the various conditions studied, a more potent product can be obtained by ultra-violet than by cathode ray treatment and that short ultra-violet light exposures, 15 seconds to 3 minutes, are at least as effective as 30 minute exposures.

Since a very potent product is obtained by ultra-violet radiation

an experiment was tried to see whether subsequent cathode radiation had any effect on the potency. It was found that the product had the same potency as with cathode radiation alone, indicating that cathode radiation destroys some of the antirachitic activity.

Spectrographic studies of the effect of cathode rays on ergosterol show that it undergoes the same change in the absorption spectrum as with ultra-violet light irradiation. Although the spectrographic results show that cathode rays bring about the same change in the spectrum of ergosterol as does the ultra-violet light irradiation, the question as to how cathode rays produce their action cannot as yet be answered. The question arose as to whether cathode rays produce their effect through production of ultra-violet light, but yeast, cholesterol, and ergosterol, when placed behind a quartz plate which is bombarded by cathode rays, fail to develop antirachitic properties. In another experiment ergosterol was spread out in a thin layer on both sides of a quartz plate to see whether cathode rays could produce sufficient ultra-violet light upon the ergosterol on the one side of the plate to activate that on the other side. The result was negative. These experiments, as far as they go, do not indicate that cathode rays produce their effect through production of ultra-violet light rays.

The above results, together with those of other workers in this field, are consistent with the theory that under the influence of ultra-violet light or cathode ray, vitamin D is being destroyed at the same time that it is being produced and that the destruction is more rapid under the influence of the cathode ray.

THE DURATION OF THE EFFECT OF ULTRA-VIOLET RADIATION ON CHICKENS.

By WALTER C. RUSSELL, O. N. MASSENGALE, AND C. H. HOWARD.

(From the Departments of Agricultural Biochemistry and Poultry Husbandry, New Jersey State Agricultural Experiment Station, Rutgers University, New Brunswick.)

Each of four lots of 2 week old chicks on a leg weakness-producing ration was given a single exposure to the filtered radiations from a quartz mercury lamp of 45, 90, 180, and 270 minutes, equiva-

lent to 15, 30, 60, and 90 minutes of direct radiation, respectively. One control lot received no ultra-violet light and another received a 15 minute direct dosage, daily. The percentage of bone ash of the femurs and large wing bones, and blood calcium and phosphorus of five to seven birds from each lot were determined at half weekly, and later at weekly intervals, throughout the experiment.

The results indicate that the duration of the effect of the 45 minute exposure is for 1 week and of the other single exposures for 2 weeks. The calcium and phosphorus of the blood fluctuate in a manner that suggests a relationship to the process of mineral deposition in the bones.

STUDIES ON THE ORGANIC PRECURSORS OF HEMOGLOBIN.

By GEORGE F. CARTLAND AND FRED C. KOCH.

(From the Department of Physiological Chemistry, University of Chicago, Chicago.)

Attempts were made to locate and identify some of the dietary factors involved as organic precursors for hemoglobin synthesis in the albino rat. Blood volume, blood hemoglobin concentration, and red blood corpuscle counts were determined on growing rats kept on various diets. Rats could not be rendered anemic on a diet containing wheat gluten as the only protein. Substituting gluten plus tryptophane or casein for gluten, the diet being kept identical otherwise, did not increase the blood hemoglobin content of the animals.

In a second series of experiments the rats were rendered severely and constantly anemic by frequent bleedings. It was found that such animals when kept on the 10 per cent gluten diet regenerated their blood hemoglobin every 10 to 14 days. The substitution of an 18 per cent casein diet for the 10 per cent gluten diet did not increase the rate of hemoglobin regeneration. Feeding red corpuscles from beef blood did not affect this rate. The same high rate of blood hemoglobin regeneration was observed in rats kept on diets deficient in vitamin B, vitamin A, and vitamin E.

GLUCOSE IN THE URINE OF NORMAL AND NEPHRITIC SUBJECTS.

By JAMES A. HAWKINS, EATON M. MacKAY, AND DONALD D. VAN SLYKE.

(From the Hospital of The Rockefeller Institute for Medical Research, New York.)

Fermentable sugar in the urine was determined in two ways. (1) The urine was mixed for 15 minutes with 2 volumes of a 20 per cent suspension of yeast, then cleared with Lloyd's reagent. The reducing substances were then determined by the gasometric modification of the ferricyanide method reported by us at last year's meeting.[14] The decrease in total reducing substances of the urine caused by the treatment with yeast indicated the amount of fermentable sugar, proper correction being made for the small amounts of reducing material in the added yeast. (2) The CO_2 formed by similar fermentation was determined by direct analysis of the urine-yeast mixture in the Van Slyke-Neill blood gas apparatus.[15]

Both methods sufficed to determine glucose in the urine, varying from 0.005 to 2.00 per cent.

Both indicated in ordinary normal urine less than 0.01 per cent, confirming Eagle.[16] Furthermore the urine remained negative after oral administration of 1 gm. of glucose per kilo.

In a number of patients observed with the "nephrotic" or "hydremic," or "tubular" type of nephritis, characterized by gross chronic albuminuria and tendency to edema, without hematuria or hypertension, the morning urine before breakfast has shown fermentable sugar, usually from 0.050 to 0.300 per cent. After 1 gm. of glucose per kilo by mouth was given the urinary fermentable sugar increased still further, both in concentration and in output per hour. The concentration in one instance reached 1.5 per cent. The blood sugar curves are normal in these cases. The kidneys are abnormally permeable to glucose.

In the "glomerulonephritic" or "azotemic" type of nephritis

[14] Van Slyke, D. D., and Hawkins, J. A., *J. Biol. Chem.*, 1927, lxxiv, p. viii.

[15] Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, lxi, 523.

[16] Eagle, H. S., *J. Biol. Chem.*, 1926–27, lxxi, 481.

there may be no excretion of fermentable sugar during fasting, but such excretion may occur after glucose administration. Glucose excretion in such cases appears attributable not so much to abnormal permeability of the kidneys, as to the fact that retarded ability to utilize absorbed glucose[17] permits its concentration to rise in the blood to heights that would cause glycosuria even in normal subjects.

ENVIRONMENTAL AND NUTRITIONAL FACTORS AFFECTING THE GROWTH OF RATS ON DIETS CONTAINING SODIUM BENZOATE.

By WENDELL H. GRIFFITH.

(*From the Department of Biological Chemistry, St. Louis University School of Medicine, St. Louis.*)

The rate of growth of young white rats on a diet containing 2 per cent sodium benzoate, 35 per cent casein, 3 per cent sucrose, 32 per cent corn-starch, 20 per cent lard, 4 per cent salt mixture, 4 per cent cod liver oil, and yeast is below normal if the rats are kept in screened bottom raised cages. Normal growth on this diet results when access to feces and sawdust is permitted, or when glycine is added to the diet. Rats which are allowed to eat feces and sawdust are more able to detoxicate sodium benzoate, either because glycine is obtained from bacterial proteins in feces or because such rats are in better nutritive condition due to the availability of additional dietary factors in feces or sawdust. In either case, access to feces and sawdust is equivalent to the addition of glycine to the diet.

LIGNIN AS A FACTOR IN THE FORMATION OF HIPPURIC ACID.

By FRANK A. CSONKA, M. PHILLIPS, and D. BREESE JONES.

(*From the Protein and Nutrition Division, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington.*)

The urine of herbivorous animals contains normally rather large quantities of hippuric acid, the origin of which is yet unknown. Lignin, which is present in comparatively large amount in the

[17] Linder, G. C., Hiller, A., and Van Slyke, D. D., *J. Clin. Inv.*, 1925, i, 247.

normal food of these animals, is known to contain an aromatic nucleus. Several investigators, among them Harten, Weiske, and others, have suggested that lignin probably furnishes the phenyl radical involved in the formation of hippuric acid, but they all failed to prove this hypothesis experimentally. Recently Paloheimo stated that lignin reappears quantitatively and unchanged in the feces. Corroborating this, Rogozinski and Starzewska found no increase in hippuric acid elimination in sheep urine when lignin was added to the diet.

We used both herbivorous (cow) and carnivorous (dog) animals in our experiments and found that lignin added to the basal diet increased markedly the amount of hippuric acid in the urine. The Folin-Flanders method as modified by Kingsbury and Swanson was applied for hippuric acid determination. The results obtained indicate that lignin, normally present in the food, is metabolized and split, benzoic acid being one of the end-products which appears in the urine conjugated either with glycine or with glycuronic acid, or both. There is some evidence pointing to the presence of another lignin degradation product in the urine, which is now being investigated.

THE EFFECT OF SUPPLEMENTARY FEEDING OF CARBOHYDRATES AND OF FAT UPON THE COMPOSITION OF HUMAN MILK.

By ISRAEL S. KLEINER and MARION BELL.

(From the Department of Physiological Chemistry of New York Homeopathic Medical College and Flower Hospital, New York.)

The chief object of the investigation was to determine whether supplementary feeding of different carbohydrates would have any effect upon the volume or composition of milk. The milk of 88 normal mothers was obtained on the 5th and 9th days post partum and analyzed. The subjects were divided into four groups as to diet: one on ordinary mixed hospital diet; the others receiving, in addition, 60 gm. of rice or tapioca, or 100 gm. of 40 per cent cream, respectively per day. Thus the two high carbohydrate groups were compared with a normal diet and with one rich in fat.

No marked effects of these supplementary feedings were seen. The variations in composition of the milk of individuals on any

one diet were so much greater than the differences in the respective group averages that it is difficult to draw conclusions. Such slight average differences as were shown may, however, reflect an influence of the diet. The volumes of milk were greatest in the carbohydrate groups and least in the fat group. Protein was highest in the control group, which of course had the largest percentage of protein in the diet. Lactose was higher in the carbohydrate and fat groups on the 5th day than in the control group, but was about on the same level in all on the 9th day. Fat was increased by the fat diet on the 5th day but averaged about the same in all groups on the 9th day. No influence whatever was seen in the average values for ash or specific gravity.

The accepted view that during the early period of lactation protein decreases and fat and lactose increase was not found to be invariably true. The protein usually decreased but an appreciable number of cases showed no increase in fat and lactose.

Later specimens of milk of many of these mothers were also analyzed. The averages for protein showed a constant but slower decrease after the 9th day and the deviations from the average became less. The lactose tended to become constant and here also fluctuations narrowed. Fat continued to increase and to show marked variations between individuals.

A careful study of the data failed to show any relationship between the composition of the early milk and the gain or loss of weight of the infant.

THE CALCIUM AND PHOSPHORUS BALANCES OF LACTATING WOMEN.

By HELEN A. HUNSCHER.

(From the Nutrition Research Laboratories of the Merrill-Palmer School and the Children's Hospital of Michigan, Detroit, and the Home Economics Department of the University of Chicago, Chicago.)

10 day calcium and phosphorus balances were determined on two lactating women at the close of a year's milk production. Their food consumption represented voluntary selection as to quality and quantity. Previous to this study, during their lactating cycles, one woman's daily output of milk had varied from 40 to 113 ounces in the 24 hour day; the other woman's, from 23 to

88 ounces. The intake of calcium and phosphorus was very high in both cases. In spite of the large output of milk over a long period of time, both subjects showed positive calcium and phosphorus balances.

CALCIUM AND PHOSPHORUS METABOLISM IN DAIRY COWS.

III. THE ADEQUATE RATION FOR HIGH PRODUCING COWS AND THE EFFECT OF EXERCISE ON CALCIUM, PHOSPHORUS, AND NITROGEN BALANCES.

By WILLIAM A. TURNER and ARTHUR M. HARTMAN.

(From the Experiment Station, Bureau of Dairying of the United States Department of Agriculture, Beltsville, Maryland.)

Two Holstein cows, 6 years of age and giving about 28 and 22 kilos of milk daily, were fed for 4 weeks a ration consisting of well cured alfalfa hay, mangel beets, and a good grain mixture (wheat bran 30, corn-meal 20, linseed oil meal 10, sodium chloride 1, disodium phosphate 5). The ratio of calcium to phosphorus in the ration was 1.1:1. The cows ate less than their energy requirements (95 per cent, according to Savage). The calcium and phosphorus balances were slightly negative, the nitrogen balances positive. Their assimilations were good. It is apparently very difficult to prevent losses of calcium and phosphorus from the bodies of high milking cows in the early stages of lactation.

During the succeeding 3 weeks the cows ate the same ration in somewhat greater quantity (105 per cent, according to Savage). During this period they were given a half hour of exercise daily. With increased calcium intake the negative balances continued (one the same and the other still more negative). The calcium assimilations were diminished. The increased phosphorus intake was apparently reflected in improved phosphorus balances and assimilations. The effect on the nitrogen metabolism was marked. With increased nitrogen intakes strongly positive balances became negative and assimilations were decidedly diminished and the excretion of nitrogen in the urine and feces was considerably increased. Apparently the demands of exercise were too great even for the excess of nutrients which the animals received and resulted in a destruction of body protein. This excessive usage of

nitrogen as a result of a small amount of exercise is rather surprising in view of results obtained in the past. It may be that when there is a shortage in the energy supplied, heavy milking cows are compelled to draw upon their body protein to meet the demand for lactose in milk secretion and that this condition is accentuated when the draft of exercise is superimposed upon that of milk secretion. It is natural to assume that this demand might affect the body protein rather than the body fat if it is admitted that fat cannot be converted into carbohydrate.

THE ACID-BASE BALANCE IN ANIMAL NUTRITION.

IV. THE EFFECT OF MINERAL ACID INGESTION BY SWINE THROUGH THREE GENERATIONS.

By ALVIN R. LAMB and JOHN M. EVVARD.

(From the Iowa Agricultural Experiment Station, Ames.)

We have previously shown the remarkable tolerance of swine to strong acid ingested with the ration, where as much as 500 cc. of N sulfuric acid were given without apparent harm over the greater part of a 10 month period.[18] In a later experiment growing swine tolerated 500 cc. of N acid per day, and made gains in weight at above the average rate, but refused to consume 1000 cc. per day.[19]

The effect of such a régime upon reproduction and succeeding generations being of importance, two lots of four sow pigs each were fed a ration of yellow corn, tankage, corn oil cake meal, alfalfa meal, and salt. One lot received 200 to 250 cc. of N sulfuric acid per head per day. Both lots reproduced normally. At the end of the suckling period five average sow pigs were selected from the young in each lot and continued on the same ration, the acid being started at 50 cc. per day and gradually increased to 300 cc. of N solution per day. These animals were bred and produced the usual number of young, the acid-fed lot producing a slightly higher percentage of vigorous young.

Average individuals in each lot were again selected and continued under the same experimental régime. These sows produced

[18] Lamb, A. R., and Evvard, J. M., *J. Biol. Chem.*, 1919, xxxvii, 317.

[19] Lamb, A. R., and Evvard, J. M., *Iowa Agric. Exp. Station, Research Bull. 71*, 1921.

and suckled two litters of young. Both lots performed apparently normally, and the experiment was finally concluded after 3¾ years of acid feeding.

The plasma bicarbonate by the Van Slyke gasometric method on the sows of the first generation averaged somewhat higher for the control lot than for the acid-fed lot. This did not interfere with growth, well being, or reproduction. There was never any evidence of pathology in the urine. The plasma bicarbonate of the sows of the third generation was slightly higher for the acid-fed lot.

Postmortem examination of the second and third generation animals showed no macroscopic effects of acid feeding on any of the viscera. In one case histological examination showed a mild fatty degeneration in the tubules of the kidney of an acid-fed animal. The breaking strength of the radius and ulna and the ribs of the second and third generations showed no variation between the lots greater than within the lots. In a few cases the bones of the acid-fed animals showed greater strength. These bones were generally more flinty in texture than the controls. The calcium and phosphorus content of the bones (humerus, radius, ulna, scapula, and skull) showed no differences in favor of either the acid-fed hogs or the controls.

Three generations of rats were produced with no apparent ill effect and optimum reproduction on a satisfactory grain, casein, and salts ration which contained a potential excess of 15 cc. of N acid per 100 gm. of feed. This ration was continued for a period of 16 months.

THE RÔLE OF THE LIQUID JUNCTION POTENTIAL IN THE ELECTROMETRIC DETERMINATION OF SINGLE ION ACTIVITY COEFFICIENTS.

By WILLIAM C. STADIE AND EFFIE ROSS HAWES.

(From the John Herr Musser Department of Research Medicine, University of Pennsylvania, Philadelphia.)

We present in this paper measurements of the activity coefficients of the bicarbonate and the carbonate ions at ionic concentrations of 0.01 to 5 M. The experiments were projected primarily to elucidate the rôle of the liquid junction potential in the determina-

tion of single ion activity coefficients, the increasing importance of which obligates us to be aware of the theoretical and experimental limitations placed on their measurement. The concentration cell offers the only method for this. Since, however, every concentration cell has a liquid junction and every liquid junction gives a potential, we encounter at once a circular situation. We cannot measure the activities of the components of the cell without knowing the liquid junction potential nor can we measure the liquid potential without knowing the activities.

We are thus compelled to use one of several indirect methods, each of which involves an assumption of doubtful character. These assumptions are: (1) The activity coefficient is dependent solely on the ionic strength. This is thermodynamically erroneous as Brönsted has shown. In dilute aqueous solutions, the assumption serves as a first approximation but to extend it to protein solutions involves serious error. (2) In solutions of KCl the activity coefficient of potassium is equal to that of chlorine. This assumption of MacInnes, upon which the table of individual ion activity coefficients of Lewis and Randall is calculated, while narrower in scope, is the same character as the first assumption. There is a high probability that it holds up to 0.1 M as Scatchard has shown. Beyond that its validity is doubtful. (3) The Bjerrum extrapolation is the liquid junction potential. The Bjerrum extrapolation has no sound theoretical basis. At best, perhaps, the extrapolation can be regarded as reflecting in a comparative way the magnitude of the liquid junction potential of any two pairs of solutions. (4) The insertion of a saturated KCl bridge eliminates the liquid junction potential between any two solutions.

Our experiments presented in this paper were designed to test the validity of the fourth assumption in the determination of the activity coefficients of the bicarbonate and carbonate ion. We therefore, determined first the activity coefficients of the bicarbonate ion in 0.01 M concentration through a range of NaCl concentration from 0 to 5 mols per liter and second the activity coefficient of the carbonate ion at 0.01 M through the same salt range. We used a hydrogen electrode as our concentration cell to measure single ion activities, *viz.* a_{H^+}, and employing one assumption, *viz.* that a saturated KCl bridge eliminates the liquid junction potential, we calculated the coefficients for HCO_3^- and $CO_3^{=}$.

The paper of Walker, Johnston, and Bray on carbonate-bicarbonate equilibria at low CO_2 pressures gives not f_{HCO_3} or f_{CO_3} but the ratio $\frac{f_1^2}{f_2}$ determined under precisely the same conditions as f_1 and f_2 in our experiments but quite independent of liquid junction. We therefore make the comparison of the two types of data by this ratio.

There is close agreement of the ratio $\frac{f_1^2}{f_2}$ through all concentrations of NaCl and identity of the $\frac{K_1}{K_2}$ ratio. The theory of concentration cells gives the following summary of liquid junction relations.

To calculate	we assume
K_1	$L_s = L_0$ and a_H (standard) known
f_1	$L_1 = L_0$
K_2	$L_s = L_0$ and a_{H^+} (standard) known
f_2	$L_1 = L_0$ and $L_2 = L_0$
$\frac{K_1}{K_2}$	Nothing.
$\frac{f_1^2}{f_2}$	$L_1 = L_2$

L_s = liquid junction potential standard reference acid *vs.* saturated KCl.
L_1 = " " " $NaHCO_3 \cdot NaCl$ *vs.* saturated KCl.
L_2 = " " " N_2CO_3 *vs.* saturated KCl.
L_0 = " " " H_2O *vs.* saturated KCl.

The comparison of our data with those of Walker, Bray, and Johnston proves nothing with respect to liquid junction potentials beyond the agreement of data obtained by two independent means. This is precisely the result expected from our previous statement that thermodynamics denies the possibility of escaping the indissoluble circle of liquid junction potential and single ion activities.

Our experiments show that the hydrogen electrode is a reversible source of E.M.F. and that we are measuring a_{H^+}, and together with evidence from the experiments of Warburg, and Hastings and Sendroy establish the following pertinent facts about H_2CO_3 systems. (1) The first and second dissociation constants obtained electrometrically and by conductivity are in close agreement. (2) The limiting slopes of the $\log f_1 - \sqrt{\mu}$ and $\log f_2 - \sqrt{\mu}$ curve

are identical with the theoretical values of the Debye-Hückel theory. (3) The form of these curves is that given by the Debye and Hückel theory for ions of small size.

This evidence makes it highly probable although by no means certain that the salt bridge, except in very acid solutions, makes the liquid junction potential small and probably less than 1 millivolt. In other words, the assumption of zero liquid junction potential is a close approximation.

PHYSICOCHEMICAL METHODS OF CHARACTERIZING PROTEINS.

VIII. THE APPARENT DISSOCIATION CONSTANTS OF PROTEINS CALCULATED FROM THEIR SOLUBILITIES AND ACTIVITY COEFFICIENTS IN CONCENTRATED SALT SOLUTIONS.

By EDWIN J. COHN and ARDA ALDEN GREEN.*

(From the Department of Physical Chemistry in the Laboratories of Physiology, Harvard Medical School, Boston.)

The solubility of certain slightly soluble proteins is increased by relatively low concentrations of neutral salts, while these and most other proteins are precipitated from solution by higher concentrations of neutral salts. The solubility of a saturating salt in a solution of given ionic strength is defined by the Debye-Hückel equation:

$$-\log \gamma = \log \frac{S}{S_0} = 0.5\, z_1 z_2 \frac{\sqrt{\mu}}{(1 + \kappa b)} - K s \mu \qquad (1)$$

The first term on the right hand side of this equation describes the increase in solubility of a slightly soluble strong electrolyte of valence $z_1 z_2$ upon the addition of a neutral salt of ionic strength μ. It has previously been employed in describing the solubility of oxyhemoglobin in dilute phosphate solutions.[20] The last term in the equation applies equally to electrolytes and non-electrolytes.[21] In sufficiently concentrated salt solutions the first term approaches

* Fellow in Medicine of the National Research Council.

[20] Cohn, E. J., and Prentiss, A. M., *J. Gen. Physiol.*, 1927, viii, 619.

[21] Debye, P., and McAulay, J., *Physik. Z.*, 1925, xxvi, 22.

a constant value. Under these conditions the salting out term suffices to describe solubility, and the equation may be written:

$$\log S = \left[\log S_0 + 0.5\, z_1 z_2 \frac{\sqrt{\mu}}{(1 + \kappa b)}\right] - Ks\mu = \beta - Ks\mu \qquad (2)$$

In concentrated solutions the logarithm of the solubility of a protein is inversely proportional to the molecular concentration or to the ionic strength. β depends not only upon the nature of the protein and of the neutral salt, but also upon the temperature and the pH.

Values of Ks and of β have previously been calculated[22] from Sørensen's measurements upon egg albumin in concentrated ammonium sulfate solutions.[23] If the salting out term is expressed as related to the molecular concentration, Ks equals 3.58; if it is related to the ionic strength per liter, Ks equals 1.19. The logarithm of the hypothetical solubility in the absence of salt, β, varies from 6.8 to 7.7 as the pH varies from 4.5 to 5.3. This change in solubility is described by assuming that egg albumin is dissociating in this range as though it were a divalent acid:

$$\frac{S}{S_0} = 1 + \frac{K_{a1} K_{a2}}{a_{H^2}} \qquad (3)$$

If the antilog of β be plotted against $\frac{1}{a_{H^2}}$ a straight line results, the slope of which yields the product of the constants of the dissociating acid groups; for these measurements upon egg albumin, 2.25×10^{-10}. Were the two dissociation constants identical the constant of each would be 1.5×10^{-5}, and coincide with the isoelectric point of egg albumin.

The solubility of hemoglobin has now been studied in solutions of different salts at 25°, and in phosphate buffer solutions differing in temperature, pH, and ionic strength. The logarithm of the solubility of the hemoglobin at constant pH was in all cases inversely proportional to the ionic strength when the latter was sufficiently great, and was defined by equation (2). The salting

[22] Cohn, E. J., *Physiol. Rev.*, 1925, v, 349.

[23] Sørensen, S. P. L., and Høyrup, M., *Compt. rend. trav. Lab. Carlsberg*, 1915–17, xii, 213.

out constant, *Ks*, has been determined for carboxyhemoglobin in ammonium sulfate solutions, in phosphate solutions, and in sodium citrate solutions. *Ks* has the same value, 1.00, for oxyhemoglobin and carboxyhemoglobin in phosphate solutions at 0°, and for the latter at 25° and at all reactions between pH 6.6 and 7.4. The value of β varies however with temperature and with pH. The variation with pH may be described by assuming that carboxyhemoglobin dissociates as a divalent acid, the product of whose constants is 1.03×10^{-14}.

THE ISOELECTRIC POINT OF CRYSTALLINE UREASE.

By JAMES B. SUMNER and DAVID B. HAND.

(From the Department of Physiology and Biochemistry, Cornell University Medical College, Ithaca.)

In an attempt to determine the isoelectric point of crystalline urease by the method of electrophoresis, it was discovered that recrystallized urease is largely precipitated by phthalate buffer at pH 4.8 to 4.4. Accordingly, we abandoned the method of electrophoresis, thinking that it would be an easy matter to find the point on the pH scale at which urease is least soluble in the phthalate buffer and to take this as the isoelectric point.

Urease is first precipitated by 0.02 M phthalate buffer on the acid side of pH 5.34 and is almost completely precipitated between pH 4.6 and 4.0. In order to try the effect of a still more acid reaction we next employed acetic acid-acetate buffer, but we were surprised to find that no urease at all was precipitated at any pH that could be obtained by mixing acetic acid and sodium acetate. Hence, it became evident that the precipitation of urease by phthalate buffer is not, as we had thought, due to the hydrogen ion concentration alone, but to the phthalate ions at certain pH values. In our experiments we have made quantitative determinations of the urease both in the supernatant liquid, after centrifuging, and in the precipitate. The precipitate has been dissolved in neutral phosphate before testing. Kjeldahl determinations have shown that the total nitrogen accompanies the urease activity. The pH values have been obtained by use of the quinhydrone electrode.

We have obtained a second curve by precipitating urease with 0.008 M lead acetate in 0.12 M acetic acid-acetate buffer. Urease

is first precipitated by lead ions in solution less acid than pH 4.63, and at pH 5.7 its precipitation is practically complete. In our experiments about 13 per cent of the urease was inactivated by the lead. The phthalate buffer destroyed no urease until the acidity became as acid as pH 4.07. At pH 3.8, in acetic acid-acetate buffer, urease is still able to decompose urea.

The curves obtained by precipitating urease by phthalate and by lead acetate cross at pH 4.95. This is probably the isoelectric point of urease. At this point about 15 per cent of the urease is capable of uniting both with lead ions and with phthalate ions. It is interesting to note that the isoelectric point of urease is far to the acid side of the point of optimum activity, which we have previously found to be at pH 7.0.

STUDIES OF THE BROMIDE AND CHLORIDE DISTRIBUTION IN THE BLOOD OF DOGS AND THE PRODUCTION OF EXPERIMENTAL EDEMA BY SODIUM BROMIDE ADMINISTRATION.

BY A. BAIRD HASTINGS AND H. B. VAN DYKE.

(From the Department of Physiological Chemistry, University of Chicago, Chicago.)

1. A study has been made of the distribution of bromide and chloride in the blood of dogs. When NaBr was added to blood *in vitro* the ratio $\frac{\text{(Br) cells}}{\text{(Br) serum}}$ is greater then $\frac{\text{(Cl) cells}}{\text{(Cl) serum}}$ at constant pH. When NaBr was fed, very high ratios of $\frac{\text{(Br) cells}}{\text{(Br) serum}}$ were encountered. In some instances $\frac{\text{(Br) cells}}{\text{(Br) serum}} = 2.0$ and the corresponding $\frac{\text{(Cl) cells}}{\text{(Cl) serum}}$ 0.5.

2. Edema has been consistently produced in the dogs fed large amounts of NaBr. This has been especially pronounced when NaCl was administered for several days prior to the NaBr. Pitting on pressure, retention of water, and increase in weight accompanied the edema. Chemical studies of the blood and urine before and after the onset of the edema have been made.

STUDIES ON THE OXIDATION-REDUCTION POTENTIAL OF BLOOD.

By MARTIN E. HANKE AND J. A. TUTA.

(*From the Department of Physiological Chemistry, University of Chicago, Chicago.*)

In view of the work of Clark and others on the oxidation-reduction potentials of several biological systems, it seemed significant to perform some potentiometric determinations directly on blood. In general the procedure was to immerse in the blood a shiny platinum electrode, to make a liquid junction from the blood to a calomel cell, and then to read the potential difference between the platinum and the mercury. Determinations were made on freshly drawn blood and serum in glass vessels, and also on circulating blood in the living animal. For the latter, dogs under barbital anesthesia, with heparin as anticoagulant, were used. In some experiments the platinum electrodes were kept in glass cannulæ inserted in the jugular or femoral veins; in others, platinum wires were inserted into the blood vessels by piercing directly the wall of the intact vessel. The liquid junction to the calomel cell was made either from the blood directly or through the skin of the animal. The results obtained by any of these techniques *in vivo* were the same.

The readings in over 10,000 observations on over 100 animals with a variety of platinum and gold electrodes varied over a range of 120 millivolts, from 0 to −120 as observed with the saturated calomel electrode, which becomes +246 to +126 with the normal hydrogen standard. 90 per cent of the readings lay within a range of 70 millivolts, and 60 per cent of the readings within a range of only 30 millivolts; that is, from +186 to +156 on the normal hydrogen scale. Any one electrode gave readings within a narrow range of 10 or 20 millivolts even in the blood of different animals, but different electrodes gave the wider variations mentioned.

These results show that blood is very poorly poised. The question of whether or not blood is partially poised within the above mentioned range might be answered by comparing the range of the blood readings with those observed with some known unpoised solutions under comparable conditions. In general it

was found that a variety of electrodes simultaneously immersed in the same unpoised solution at the pH, temperature, and oxygen tension of normal blood, showed variations as great as 200 millivolts, or about twice the range of variation observed with blood. And in particular was it found that two electrodes, reading 50 to 100 millivolts different in an unpoised phosphate buffer at pH 7.4, would read only 10 to 30 millivolts different when present in blood. The results indicate that blood is better poised than a buffer solution at the same pH and oxygen tension.

The effect of the injection of dissolved oxidizing and reducing agents on our potentiometric readings on circulating blood was studied. With oxidizing agents, *e.g.* 1 cc. of 0.1 N $KMnO_4$ per kilo intravenously injected, there follows a prompt increase in positive potential of 60 to 100 millivolts and then a gradual return in about 15 minutes to the original normal value. With reducing agents, *e.g.*, 0.1 N hydrosulfite, there is a sudden increase in negative potential of over 200 millivolts, and a prompt return to normal in 3 to 5 minutes. A number of the oxidation-reduction indicator dyes of Clark were similarly studied. The injection of oxidized methylene blue, 1 cc. of 0.5 per cent aqueous solution per kilo, causes a prompt increase in negative potential of 50 to 80 millivolts, with a gradual return to normal. We believe that this increase in negative potential is caused by reduced methylene blue which is formed by the action of tissues on the injected oxidized methylene blue. The results with other dyes were very slight and inconsistent. We believe that the unique effect of methylene blue is due to the fact that it combines a suitable permeability for tissues with an E_0 value, not too highly reducing, so that the tissues can extensively reduce it, and yet reducing enough to be much more reducing than blood.

The significance of our potential readings on blood is intimately related to the general question of the significance of readings of unpoised solutions. We believe that in an unpoised solution the observed potential is largely determined by the following two factors: (1) an inherent tendency on the part of the electrode to have a particular value, and (2) dissolved oxidizing and reducing agents like oxygen, which only partially or ineffectively get into equilibrium with the electrode, which are designated as semireversible systems by Clark. There may be overlapping of these factors,

and in the presence of oxygen the variations between different electrodes represent chiefly the varying extent to which they get into equilibrium with oxygen. To what extent the relative uniformity of our readings on blood is determined by dissolved oxygen, or whether there are present other oxidizing and reducing agents which partially and ineffectively get into equilibrium with the electrode, remains to be determined by a further general analysis of these factors, and also by determination of the readings given by blood in the absence of oxygen.

It appears then that blood is very weakly poised. Perhaps, however, it may be considered to be partially poised within a certain range, since a variety of electrodes, although diverging widely in an unpoised solution at the same pH and oxygen tension as blood, will give readings closer together when in blood, and also since there is a prompt and consistent return of the reading to the original value, after being temporarily deflected by the injection of either oxidizing or reducing agents.

A NEW METHOD FOR THE DETERMINATION OF CYSTEINE AND CYSTINE IN THE PRESENCE OF BLOOD AND TISSUE.

By V. B. DOWLER.

(From the Section on Chemistry, The Mayo Foundation, Rochester, Minnesota.)

Cysteine is not oxidized to cystine with iodine, provided but a small amount of cysteine is present and further provided the cysteine is added to the solution of iodine. Under these conditions, cysteine is quantitatively oxidized to products other than cystine, probably to cysteic acid.

Under precisely similar conditions cystine is not at all acted upon by iodine. These chemical properties of cysteine and cystine are the basis of a new method for the determination of these sulfur derivatives when both are present in a solution; the method is based upon the previous work of Folin and Looney, and of Hunter and Eagles. These investigators determined cystine through its effect on an alkaline solution of phosphotungstic acid in the presence of sodium sulfite. The method for the determination of cysteine and cystine in the presence of blood and tissue is as follows: The protein is precipitated by the Folin-Wu tungstic acid

method. One-half of the filtrate is neutralized and made faintly alkaline with ammonia; a trace of ferric chloride is added. The solution is then aerated for 1 hour, in order to convert all the cysteine to cystine. The solution is made 0.01 N acid and is poured into a few cc. of a solution of 0.01 N iodine. The excess iodine is reduced with a few drops of a dilute solution of sodium sulfite and the color is determined by the addition of sodium hydroxide, sodium sulfite, and the uric acid reagent in proper volumes. The other half of the Folin-Wu filtrate is poured into a few cc. of a solution of iodine. It is allowed to stand for 15 minutes. The excess iodine is reduced. This solution is then made faintly alkaline as before and is aerated the same length of time. The cystine present is then determined as before. The difference between the two amounts of cystine present gives the amount of cysteine. Cysteine and cystine can be quantitatively recovered after addition to blood and tissue by this method.

Many samples of cysteine have been examined and have been found to contain as much as 30 or 40 per cent of cystine. These were prepared by the usual method for the preparation of pure cysteine.

The preparation of cysteine is a difficult matter; when cystine is reduced with tin and hydrochloric acid, the solution of cysteine after removal of the tin, may contain not more than 3 per cent of cystine; if this is allowed to stand exposed to air, even in the presence of 0.3 N sulfuric acid, the cysteine is oxidized. If the solution is evaporated to dryness in a vacuum and the cysteine-hydrochloride is isolated as a dry powder, it rapidly oxidizes to cystine. These results agree with the observations of Andrews on the optical activity of cysteine and cystine. Cystine is slowly and incompletely reduced in the presence of fresh liver or muscle. These results emphasize the significance of the -SS grouping in biological oxidation.

THE OXIDIZING AND REDUCING POWER OF CYSTEINE AND GLUTATHIONE.

By EDWARD C. KENDALL.

(From the Section on Chemistry, The Mayo Foundation, Rochester, Minnesota.)

Kendall and Nord have shown that cysteine cannot reduce indigo carmine and cystine cannot oxidize reduced indigo, except in the presence of an activating substance. This third component of the system can be prepared from indigo carmine by the action of hydrogen peroxide or sodium disulfide. Dixon and Tunnicliffe investigated this reaction and apparently showed that indigo carmine was readily reduced with cysteine, in the absence of oxygen. They explained the failure of Kendall and Nord by the presence of oxygen which maintained the indigo carmine in the oxidized form.

This reaction has been further investigated and it has been shown that no one of fifteen samples of indigo carmine could be reduced by any one of six samples of cysteine except in the presence of traces of oxygen or sulfur. It has also been shown that the disulfide linkage can act as an oxidizing agent when properly activated. In the presence of suitable activating agents cysteine and oxidized glutathione exert an oxidizing action which is of the same intensity as that of elemental sulfur in colloidal form. The probable chemical grouping, which is essential for the activation of the -SH and -SS groups, is an addition product of the indigo carmine which involves the double bond. It is destroyed by heat. In the absence of this activating agent, the -SH and -SS groupings are not in equilibrium with each other. Oxidized and reduced glutathione are capable of reacting with suitable groups, but their action is highly specific. *In vivo* experiments have been carried out which emphasize the importance of the -SS groupings in the physiological manifestations of cysteine and glutathione.

THE ISOLATION OF ARGININE, HISTIDINE, AND LEUCINE FROM HYDROLYZED CRYSTALLINE INSULIN AND THE ACETYLATION OF CRYSTALLINE INSULIN.

By HANS JENSEN.

(*From the Department of Pharmacology, the Johns Hopkins University, Baltimore.*)

The first part of this work has been published in the *Journal of Pharmacology and Experimental Therapeutics.*[24] The acetylation of insulin has been carried out in the cold and with heat. The product obtained by allowing insulin to stand with acetic anhydride at room temperature for 24 hours is insoluble in 10 per cent acetic acid but soluble in alkali. The activity of this product is about one-fifth of the original. The product which was obtained by heating insulin with acetic anhydride for 2 minutes, is insoluble in acid and alkali. Further experiments are being carried on at the present time in this direction.

RESPIRATORY QUOTIENT STUDIES WITH SYNTHALIN.

(A DIGUANIDYL DECAMETHYLENE.)

By WALTER G. KARR, O. H. PETTY, and C. SCHUMANN.

(*From the Biochemical Laboratories of the Graduate School of Medicine of the University of Pennsylvania, and the Department of Metabolic Diseases, Philadelphia General Hospital, Philadelphia.*)

A study was made in four diabetics of the respiratory quotient, blood sugar, and urine sugar after a glucose meal. Readings of the fasting respiratory quotient and blood sugar were taken and 0.5 gm. of glucose per kilo was given by mouth. The respiratory quotient, blood sugar, and urine sugar were determined at intervals after this meal for a 3 hour period. This test was repeated in exactly the same manner with the exception that synthalin was given before the glucose meal. Without the drug the patients showed, after the glucose meal, little or no increase in the respiratory quotient and a typical decreased sugar tolerance. In contrast to this, when the synthalin was given, the respiratory quotient rose nearer to 1, the tolerance for sugar was increased, and in consequence

[24] Jensen, H., Wintersteiner, O., and du Vigneaud, V., *J. Pharmacol. and Exp. Therap.*, 1928, xxxii, 387.

the combustion of carbohydrate was increased during the 3 hour period. The data indicate that synthalin aids in the utilization of carbohydrates in the diabetic.

THE CONDUCTIVITY METHOD AND PROTEOLYSIS.

II. THE EFFECT OF AMINO NITROGEN ON THE CONDUCTIVITY.

By HARRY D. BAERNSTEIN.

(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison.)

The digestion of diluted egg white by pepsin and by papain was followed at several pH levels by means of the Van Slyke amino nitrogen and the conductivity methods. It was found that the two curves of digestion coincided when the pH was below 1.3 and above 6.8. Between these limits and below pH 3.00, the conductivity decreases for a time faster than the amino nitrogen increases. Then the reverse is true. Above pH 3.00 the conductivity always increases during digestion, but the two curves do not coincide until a pH of 6.8 is reached.

These changes may be duplicated by adding simple ampholytes to solutions of HCl and NaOH and noting the effects on the conductivity.

Measurements with the quinhydrone and hydrogen electrodes show that the various substances bind hydrogen ions when the pH is below 4.75, but liberate them when the pH is higher.

There is practically no change in pCl during peptic digestion as shown by the Ag, AgCl electrode. Preliminary measurements show that glycine does bind chloride ions, however.

The results seem to justify the opinion that the same mechanism of binding is adequate to account for all the changes observed, whether in digestion experiments or in the others.

Preliminary calculations on the basis of Bjerrum's theory of ampholytic dissociation give good agreement between calculated and observed values of the conductivity changes.

THE EFFECT OF NEUTRAL SALTS ON THE HYDROLYSIS OF PROTEINS BY PEPSIN.

By T. L. McMEEKIN.

(From the Physiological Chemistry Laboratory, Emory University, Georgia.)

The rate of peptic digestion of coagulated egg white as affected by the addition of anions and cations in various concentrations was studied. The pepsin used was partially purified and the rate of digestion measured as previously reported by T. L. McMeekin and F. C. Koch. The osmotic swelling of the egg white as affected by the same anions and cations also was determined. Salts of the cations Na, K, Ca, Ba and anions Cl, Br, I, NO_3, and SO_4 in concentrations greater than N/256 had inhibitory effects on peptic digestion in a hydrochloric acid medium. For equivalent concentrations the percentage depression of peptic digestion by sodium and potassium ions is very slightly less than by calcium and barium ions. The effect of anions is more variable. Comparison of the percentage depression in digestion and in osmotic swelling respectively shows a marked parallelism. The order of depression in digestion by equivalent concentrations is Cl < SO_4 < Br < NO_3 < I, while in osmotic swelling it is Cl < Br < SO_4 < NO_3 < I. The salts have no effect on the pepsin itself nor do they interfere with the adsorption of pepsin by egg white at a pH of 1.2.

THE QUANTITATIVE ESTIMATION OF DIGESTIVE FUNCTION.

By OLAF BERGEIM.

(From the Laboratory of Physiological Chemistry of the University of Illinois, College of Medicine, Chicago.)

The gastrointestinal digestive functions are primarily enzymatic. Quantitative estimations of enzyme action can only be carried out by determining the extent of change brought about under definite conditions in the presence of an excess of substrate. It would seem therefore that in spite of the slight use made of it either clinically or experimentally the correct principle in the quantitative estimation of gastrointestinal digestive functions is to feed a considerable "overload" of suitable foods and to determine the extent of digestion of such foods by fecal analysis or in certain cases by gastric analysis. The ordinary utilization experiment, of little

value for this purpose, is modified through the selection of suitable difficultly digestible proteins, fats, and carbohydrates and through the use of the simplified type of utilization experiment previously described. Some of the difficulties encountered are discussed and application of the procedure to the study of starch and protein digestion in avitaminoses is shown.

A METHOD FOR STUDYING THE DISTRIBUTION OF FECAL LIPIDS.

By WARREN M. SPERRY.

(*From the Department of Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, New York.*)

The primary purpose of this investigation is to determine the rôle played by bacteria in the fecal lipid excretion, which has been found to occur in dogs on a lipid-free diet. The method is a much modified form of the Strasburger procedure of fractional centrifugation. The feces are collected in weekly periods under isotonic sodium chloride containing 1:1000 parts of mercuric chloride. Immediately after the last collection the mixture is stirred vigorously for several hours with a power stirrer and filtered through cheese-cloth to remove the ever present hair, which is washed many times with isotonic sodium chloride and analyzed as a separate fraction. The suspension is diluted to a volume of 1 to 2 liters, depending on the amount of feces, and allowed to settle 1 hour. The supernatant liquid is removed by suction, the settled material shaken up with 500 to 900 cc. of sodium chloride solution, and allowed to settle an hour, after which the top layer is removed as before and added to the first suspension. The solids at the bottom are examined under the microscope in suspension and are usually found to be nearly bacteria-free. The suspension is now shaken up and allowed to settle 2 hours, the residue washed as above and allowed to settle for 2 hours, examined under the microscope, and if found nearly bacteria-free, as is usually the case, added to the first residue. This process is repeated, a longer period being allowed for settling and the volume of the suspension being increased by the "washings" each time, until a period of 2 days is reached. Frequently after one and almost always after two 2 day periods the suspension is found to contain little else than

bacteria. The number of settling periods and their length vary with the experiment, depending somewhat on the amount of feces and the apparent speed of settling. Altogether between 20 and 30 settling periods are usually found necessary. All stages are followed carefully by microscopical examination, a procedure which has been found to be absolutely essential because of the variation in samples of feces. The end result is two fractions, one made up almost entirely of non-bacterial substance and the other a suspension which is estimated to be at least 80 per cent and probably over 90 per cent bacterial in nature.[25] Complete separation is not possible because some large bacteria have greater sedimentation rates than a great many of the non-bacterial particles, and some of the non-bacterial particles have slower sedimentation rates than most of the bacteria.

The bacterial suspension, which has a volume of 4 to 9 liters, is concentrated in a vacuum to a little less than a liter, and centrifuged until the supernatant liquid is clear. The bacteria are washed with a salt solution having a concentration (estimated from volumes before and after concentration) the same as that in which they were suspended at the end of the concentration. The packed bacteria are then examined under the microscope and found not to have changed in appearance.[25] The smear is stained with sudan IV to see whether any of the few amorphous particles present contain a great deal of fatty substance; but no particles have been seen to stain. The bacteria fraction is dried and washed for 5 minutes with petroleum ether to remove any fatty substances which might have been held mechanically.

The five fractions: bacteria, non-bacterial solids, soluble (including the supernatant liquors from the centrifuged bacteria and ether washings of all vessels used in the separation), petroleum ether extract of bacteria, and hair, are now analyzed for total lipids, unsaponifiable material, total fatty acids, solid fatty acids, and liquid fatty acids by methods described previously.[26]

[25] Dr. Bayne-Jones of the Department of Bacteriology of this school has kindly examined a number of typical non-bacterial fractions and smears of the packed bacteria after centrifuging and confirms me in the view that the separation is probably over 90 per cent complete.

[26] Sperry, W. M., and Bloor, W. R., *J. Biol. Chem.*, 1924, lx, 261. Sperry, W. M., *J. Biol. Chem.*, 1926, lxviii, 357; 1926–27, lxxi, 351.

The results of the sixteen experiments carried out so far are not concordant enough as far as the bacterial fraction is concerned to justify final conclusions. Values for percentage of bacterial lipids in total fecal lipids as high as 64 and as low as 14 per cent have been obtained but the majority of experiments has given percentages lying between 25 and 45 per cent. All that can be said at the present time is that bacteria do seem to play a very appreciable rôle in fecal lipid excretion and usually contribute somewhere around a third of the total. The solid non-bacterial portion contains most of the lipids which are not in the bacterial fraction, the other three fractions usually giving negligible amounts. The low lipid content of the soluble fraction is particularly interesting since here we would expect to find any soluble soaps and free lipids in suspension. The highest value obtained for this fraction was 17 per cent and thirteen of the sixteen experiments gave percentages below 10 per cent. Apparently this lipid excretion is present almost entirely in structures which can be centrifuged down from suspension and it would seem, therefore, that very little of it comes from secretions into the intestine.

THE DETERMINATION OF IODINE IN FAT AND FATTY FOODS.

By J. F. McCLENDON, DON MATHIESON, and JOHN HYNES.

(From the Laboratory of Physiological Chemistry, University of Minnesota Medical School, Minneapolis.)

There is more difficulty in the determination of iodine in food containing a high percentage of butter or other fats because of the volatility and high carbon content of the fat which, in combustion, tends to produce soot or tarry products. In order to avoid this, the fat is separated from the food, melted, and sprayed into a silica combustion tube (1″ × 2′ with elbow 1″ × 1′) by means of an atomizer, oxygen being used instead of air. (A platinum gauze or spiral is placed inside the silica tube to act as a catalyst in the combustion.) The atomizer must be warm enough to keep the fat melted but not hot enough to carbonize it. The spray end of it is therefore water-cooled. The rate of flow of the butter fat in relation to the oxygen is regulated so that there is sufficient oxygen for complete combustion and yet a rich enough mixture for rapid propagation of the flame. The silica tube is heated with

a row of ten Meker burners set close together. The elbow is fitted into an absorption vessel with dilute alkali, sealed around the elbow with wet asbestos, and immersed in ice water. The absorption flask is connected with a Cottrell precipitator and very rapid air pump.

Milk or other fatty food is either powdered or reduced to a hard mass in the form of a stick about 1 inch in diameter. In case of powder it is formed into such a stick by packing it into a paper tube. The stick is placed in a steel tube in which it loosely fits and is advanced by means of a micrometer screw. The open end of the steel tube is water-cooled. The stick is fed into the hot silica combustion tube (2″ × 2′ with elbow 1″ × 1′) so slowly that it burns without soot. The upper part of entrance of the combustion tube is closed with a tin funnel admitting oxygen. The lower edge of the funnel is bent up to admit the steel tube. It is more convenient to churn milk and analyze the butter fat and skim milk separately than to analyze whole milk powder.

The remainder of the analysis is described in the *Journal of the American Chemical Society*.[27]

DEMONSTRATION OF SMALL AMOUNTS OF CALCIUM.

By SAMUEL AMBERG and J. LANDSBURY.

(*From the Mayo Clinic, Rochester, Minnesota.*)

Solutions were prepared from ash-free gelatin containing 5 per cent gelatin and either ammonium oxalate, sodium fluoride, sodium citrate, or sodium or potassium oleate in various concentrations.

The gelatin mixtures were placed in tubes of 9 mm. inside diameter, 3 ml. to each tube. After the gel had formed, various solutions were placed on top, 1 ml. always being used. The substances added to the gelatin must be present in certain concentrations in order to give a reaction with calcium solutions placed on top. The reaction is a ring reaction. So far calcium solutions of a concentration of M/4000 have reacted with ammonium oxalate mixtures whereas those with a concentration of M/40,000 have not. With fluoride mixtures a concentration of

[27] McClendon, J. F., *J. Am. Chem. Soc.*, 1928, l, 1093.

M/400 was necessary and still greater concentration with sodium citrate mixtures. Most of the experiments were conducted with gelatin containing M/500 sodium or potassium oleate, the reaction of the mixtures being of a pH of about 7.4.

The solutions were made roughly so that the figures of the concentrations are approximate. The substances tested were lithium sulfate, sodium chloride, sulfate, and nitrate, potassium chloride, iodide, and sulfate, magnesium sulfate, strontium chloride, barium acetate, calcium hydroxide, sulfate, acetate, and oxalate. Sodium and potassium hydroxide were also tested. Redistilled water was used. If filtration is necessary it has to be done with ash-free filter paper. With exception of the hydroxide the salt solutions were brought to a pH of approximately 7.4.

The lithium, sodium, and potassium salts, including the hydroxide gave sometimes a weak reaction in a concentration of N/200 with reference to the halogen. At times there was no reaction or it was questionable. The next concentration of N/2000 was always negative. The more concentrated solutions reacted always. With magnesium sulfate the M/400 concentration always gave a positive reaction. With strontium chloride the M/4000 concentration was positive, and with barium acetate M/80,000 was positive. This concentration was also effective in most experiments with the calcium sulfate, acetate, and hydroxide. A saturated oxalate solution always reacted positively and also acted in dilutions, which have not been worked out, as yet. A concentration of M/80,000 calcium seems to be the limit of the reaction. This is 0.0005 mg. of calcium in 1 ml.

Serum from normal individuals diluted 100 times used in 1 ml. amount reacted promptly as was to be expected.

THE COLORIMETRIC ESTIMATION OF BLOOD SERUM CALCIUM.

By JOSEPH H. ROE.

(From the Department of Chemistry, George Washington University Medical School, Washington.)

The original colorimetric method of Roe and Kahn,[28] in which the serum calcium is precipitated from an alkalinized trichloroacetic acid filtrate, as $Ca_3(PO_4)_2$, and determined as phosphorus

[28] Roe, J. H., and Kahn, B. S., *J. Biol. Chem.*, 1926, lxvii, 585.

by the Benedict and Theis procedure, has been revised. The Fiske and Subbarow method for estimating inorganic phosphorus has been substituted for the Benedict and Theis technique. This modification has permitted the development of a much simpler and more rapid technique. The revised technique has been applied to a micro procedure in which 0.02 mg. of calcium can be determined accurately.

THE DETERMINATION OF LACTIC ACID IN BLOOD.

By C. FERDINAND NELSON and E. R. LEHNHERR.

(From the Department of Biochemistry, University of Kansas, Lawrence.)

Two methods are discussed. In one a Folin-Wu blood filtrate is treated with 25 per cent acetic acid and N/150 potassium permanganate. The acetaldehyde formed is distilled over and bound in bisulfite solution. The excess bisulfite is titrated with 0.1 N iodine. The bound bisulfite is liberated by means of two phosphate buffer solutions and titrated with N/150 iodine solution. The method can be carried out in the presence of sugar with an error of 3 to 5 per cent.

In the second method sugar is first removed from the blood filtrate by use of copper sulfate and calcium hydroxide (Van Slyke method). The filtrate is then treated with sulfuric acid and a colloidal solution of manganese dioxide. The acetaldehyde formed is distilled over into bisulfite and titrated as in the previous method.

FAILURE TO RECOVER CYSTINE BY COMMON METHODS OF REMOVING PROTEIN FROM BLOOD.

By T. SWANN HARDING and C. A. CARY.

(From the Research Laboratories of the Bureau of Dairying, United States Department of Agriculture, Beltsville, Maryland.)

Three 100 cc. samples of fresh blood were shaken respectively with 30, 20, and 5 mg. of dry cystine. The blood was then coagulated, treated with kaolin, trichloroacetic acid, *etc.*, as in the amino N determination in this laboratory[29] and the Folin and Looney[30]

[29] Cary, C. A., *J. Biol. Chem.*, 1920, xliii, 477.

[30] Folin, O., and Looney, J. M., *J. Biol. Chem.*, 1922, li, 421. Looney, J. M., *J. Biol. Chem.*, 1922, liv, 171.

cystine method applied to the protein-free extracts. In the first two samples 12.3 and 3.2 mg. of cystine were recovered. With the 5 mg. of cystine there was no recovery. It is likewise lost when added in solution to blood. It is not adsorbed by the kaolin or removed by the trichloroacetic acid when used as above, but is carried down in the heat coagulation of the protein. It is likewise lost when blood is deproteinized by means of ferric hydroxide or tungstic acid. Similar experiments were carried out with blood plasma from which the protein was removed by ultrafiltration. Cystine added in dilute acetic acid was not recovered, but when added in a solution of NH_4OH it was recovered quantitatively. Evidence was obtained which indicates that very little, if any, free cystine occurs in cow blood plasma. Data are given on the concentration of glutathione in blood and blood plasma obtained from cows.

A STUDY OF THE ESTIMATION OF CHLORIDE IN SERUM.

By D. WRIGHT WILSON and ERIC G. BALL.

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia.)

A comparison has been made of the determination of chlorides by means of macro methods involving alkaline ashing and the Parr bomb and the micro methods of Van Slyke and Whitehorn with certain modifications. Portions of serum were carefully ashed in platinum dishes after the addition of sodium carbonate. After solution of the ash, the chloride was precipitated with silver nitrate. Other portions were mixed with sodium peroxide in a Parr bomb and burned, after which the contents were dissolved in water and treated first with hydrazine and then with silver nitrate. These two macro methods showed suitable agreement. The Whitehorn micro method agreed well with the macro methods. Van Slyke's micro method yielded results 3 to 11 per cent low with dog and human serum. Digestion for 16 hours instead of 2 did not eliminate the error. If 1 cc. of aqueous silver nitrate was added to serum in place of the 3 cc. of $AgNO_3$-HNO_3 mixture and the 3 cc. of nitric acid added a few minutes later, and the mixture digested as usual, good agreement with the macro methods was obtained. The procedure was also shortened by digesting with gentle boiling

over a free flame for 15 to 20 minutes instead of digesting in a boiling water bath for 2 hours. The same relations held true for dog whole blood.

Tissues yielded results 9 to 15 per cent low with Van Slyke's regular procedure. The use of aqueous silver nitrate reduced the error by about one-half.

POTENTIOMETRIC DETERMINATION OF RELATIVE REDUCING RATES OF SUGARS FOR FERRICYANIDE AND IODINE.

By NOBORU ARIYAMA and PHILIP A. SHAFFER.

(From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis.)

With the object of determining the degree of activity of various sugars toward different oxidizing agents the rate of reduction of several electromotively active oxidants by sugars has been followed at known pH by measuring the rate of fall of potentials imposed upon bright platinum electrodes. It was expected that the rate of oxidation of the sugar would depend chiefly if not wholly on the potential difference between oxidant and reductant (sugar). Since the oxidizing potentials of ferricyanide (+ ferrocyanide) in strong alkaline solutions and of I_2 (+ KI) at moderate alkalinity are little affected by pH change, and are quickly registered by electrodes, the method seemed to offer a means of quantitative measurement of relative activity of the sugars.

The results indicate that under constant conditions with ferricyanide as the oxidizing agent, the rate varies greatly with individual sugars. In strong alkali the order of activity is fructose, mannose, arabinose, galactose, glucose, the last three being about alike; at lower alkalinity the rate of reduction decreases greatly until at pH 10.5 the hexoses and pentoses are oxidized only slowly at 37° by ferricyanide. But at pH 8.9, I_2-KI *of the same oxidation potential* as ferricyanide (E_h of both about + 0.520 volt) rapidly oxidizes the aldoses. The rate of oxidation is therefore *not* determined by the potential gradient between oxidant and sugar. Perhaps the difference may be attributed to the respective electron capacities of ferricyanide (1) and iodine (2).

THE CATALYTIC OXIDASE-LIKE ACTION OF METHYLENE BLUE IN SUGAR PEROXIDATION.

By BEN K. HARNED.

(From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis.)

We have previously reported that when alkaline sugar solutions are being autooxidized by molecular oxygen, a large percentage of the absorbed oxygen is activated presumably through the formation of an intermediate sugar peroxide. The activated oxygen is capable of inducing the oxidation of a number of substances such as arsenite, indigo carmine, and acetoacetate which are only slowly if at all affected by molecular oxygen.

We have since observed that the addition of very small quantities of methylene blue to a system in which glucose is inducing the oxidation of arsenite results in the absorption of twice as much oxygen and the oxidation of twice as much arsenite.

Oxidized methylene blue oxygenated in the absence of a reducing sugar has no effect upon arsenite and in the presence of a reducing sugar large amounts of the dye have scarcely any more effect than small amounts. The phenomenon is not due to the effect of heavy metals introduced with the methylene blue since cyanide does not retard the reaction and the addition of corresponding amounts of ferrous sulfate in place of the dye has no effect.

The results must depend upon the fact that methylene blue is reduced by the sugar and the leucomethylene blue is oxidized by molecular oxygen with the intermediate formation of a methylene blue peroxide which is capable of oxidizing arsenite.

In order to demonstrate the fact that leucomethylene blue forms an intermediate peroxide when oxidized, the *reduced* form of the dye was oxidized by oxygen in the presence of arsenite. The quantitative relations showed that 1 mol of the dye absorbed 1 mol of oxygen and activated one-half of the absorbed oxygen, resulting in the oxidation of 0.5 mol of As_2O_3 to As_2O_5. In the absence of another acceptor such as arsenite 1 mol of leucomethylene blue absorbs 0.5 mol of oxygen. In this case we may suppose that the leucomethylene blue peroxide first formed, oxidizes a second mol of leucomethylene blue, the total O_2 absorbed being 1 atom per mol.

From the quantitative relationships we have been able to calculate that 1 mol of methylene blue is reduced and reoxidized from twenty to forty times and possibly more. The dye thus simulates an oxidase, the importance of which in biological oxidations has long been recognized. Glutathione and other sulfuryl compounds are known to act in a similar manner under other conditions.

This only partially explains the phenomenon and does not elucidate the entire mechanism by which the amount of arsenate is doubled in the presence of the dye. This can only be explained by assuming that under the combined influence of the dye and oxygen the sugar or the sugar-arsenite complex, analogous to sugar-phosphate complexes, is more completely oxidized than under the influence of oxygen alone. This assumption is in accord with the observations of Spoehr[31] who found that glucose in disodium phosphate was not oxidized by molecular oxygen at a detectable rate. The addition of methylene blue, however, resulted in sugar oxidation with the formation of CO_2.

Methylene blue does not increase the rate of induced oxidation of all acceptors. In $Ba(OH)_2$ solutions of glucose, added methylene blue does not increase the amount of barium peroxide formed. When acetoacetate is present as acceptor its induced oxidation under some conditions is retarded by the presence of methylene blue.

THE REACTION OF BORATE AND SOME SIMPLE SUGARS.

By MILTON LEVY.

(*From the Laboratories of Biological Chemistry, St. Louis University School of Medicine, St. Louis.*)

The presence of borate interferes with the rate of oxidation of aldo sugars (glucose, galactose, arabinose, maltose) to the corresponding acid by alkaline iodine solutions except in the case of lactose. Evidence that combination occurs between borate and all of these sugars may be obtained by observation of the rotatory power in the presence of borax and the effect of the addition of an equivalent amount of a strong acid upon this rotatory power.

[31] Spoehr, H. A., *J. Am. Chem. Soc.*, 1924, xlvi, 1494.

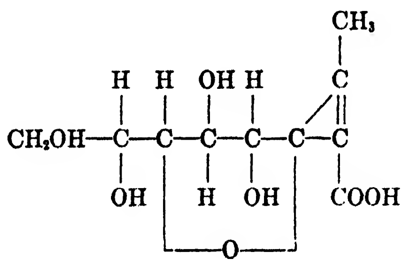
Solutions containing 0.05 M $Na_2B_4O_7$ and 0.5 M glucose, galactose, and arabinose are definitely acid to litmus; with 0.25 M maltose the solution is approximately neutral, whereas the solution containing 0.25 M lactose is still alkaline to phenolphthalein. The addition of a strong acid in equivalent quantity to a solution of borax and glucose in optical equilibrium indicates that borate combines with α-glucose but calculations show that it also combines with β-glucose, though to a slighter extent.

THE METABOLISM OF GLUCOSE CYCLOACETOACETIC ACID IN THE DOG.

By EDWARD S. WEST AND F. H. SCHARLES.

(From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis.)

In the course of the investigation of the metabolism of condensation products of acetoacetic acid and glucose in the animal body glucose cycloacetoacetic acid of the formula:



was injected subcutaneously into a dog in the form of its sodium salt in aqueous solution. Its excretion in the urine was studied polariscopically and by means of a quantitative estimation of the reduction of mercuric acetate in strongly alkaline solution. By mercury reduction 88 per cent of the compound was recovered in the urine, and this figure was confirmed polariscopically. From this it is concluded that the animal body does not metabolize glucose cycloacetoacetic acid. The compound is apparently non-toxic.

COMPARISON OF THE LACTIC ACID METABOLISM OF SMOOTH AND STRIATED MUSCLE.

By ETHEL RONZONI.

(*From the Department of Internal Medicine, Washington University School of Medicine, St. Louis.*)

Evans[32] has shown smooth muscle from various sources to be capable of producing lactic acid under anaerobic conditions, presumably from glycogen.

In the experiments here reported chicken gizzard and striated muscle were used. The accompanying table shows the phosphate, carbohydrate, and lactic acid distribution in the two types of muscle when frozen *in situ* before disturbing the circulation.

	Mg. per 100 gm. tissue.	
	Striated.	Smooth.
Total phosphate	250	130
Inorganic phosphate	110– 120	40– 50
Carbohydrates (total after acid hydrolysis)	300–1000	260–300
Glycogen	50– 750	50– 80
Free sugar	70– 90	60– 80
Non-fermentable reducing substance	195– 208	160
Hexose phosphate* (as glucose assuming hexose diphosphate)	200– 250	0
Lactic acid	30– 60	30– 40

* About one-sixth of this value is included in the determination of total carbohydrate after acid hydrolysis.

Experiments conducted under anaerobic conditions with smooth muscle, demonstrated the following: When the optimum concentration of glucose, about 200 mg., was added to the reaction mixture, Ringer's solution containing phosphate and bicarbonate at a pH 7.6, 180 to 200 mg. per cent of lactic acid were formed per hour at 38°. Without addition, from the carbohydrate which had previously been stored in the muscle, 50 to 60 mg. per cent of lactic acid were produced during the 1st hour, with a total yield of about 120 mg. per cent in 4 hours, a value not further increased. If glycogen was added, this amount was not increased. If hashed,

[32] Evans, C. L., *Biochem. J.*, 1925, xix, 1114.

the addition of glycogen allowed some increase to 80 or 90 mg. per cent. Glycogen would not be expected to diffuse into the cell until split to a diffusible form. Presumably injury due to hashing liberates from the cell enough diastatic enzyme to allow this to proceed to a limited extent. From the study of the phosphate changes there was no evidence that lactic acid was derived through the medium of hexose phosphate. If the smooth muscle was frozen before being placed in the reaction mixture, no action of any kind could be demonstrated either with glycogen or glucose.

This differed from the reactions of striated muscle. Whether frozen or hashed, the striated muscle produced 700 to 900 mg. per cent of lactic acid without carbohydrate addition. Unless the muscle happened to contain low initial carbohydrate store, the lactic acid production was not materially increased by the addition of glycogen. Glucose could not be utilized even when there was no stored carbohydrate. Moreover, it was easily demonstrable in striated muscle that lactic acid was in part derived through the medium of hexose phosphate. Experiments are now in progress to determine the reactions of intact skeletal muscle.

FREE SUGAR IN LIVER AND MUSCLE TISSUE.

BY MARSCHELLE H. POWER AND T. ALFRED CLAWSON, JR.

(From the Section on Biochemistry, The Mayo Foundation, Rochester, Minnesota.)

Comparative determinations of the reducing substances in tissue were made by three methods: (1) simple acid extraction followed by precipitation with sodium tungstate, (2) extraction with hot water, concentration, and final precipitation with phosphotungstic acid, and (3) extraction with alcohol, concentration, and precipitation with phosphotungstic acid. The reducing values of the filtrates were determined by a modified Shaffer-Hartmann method, both before and after fermentation by yeast. The type of reduction obtained by heating the sugar reagent with filtrates made by the first method was so unsatisfactory that it was abandoned in subsequent work. Filtrates made by the second method gave much higher reducing values, both before and after fermentation, than those prepared by the third method. The true glucose (fermentable sugar) by the two methods showed satisfactory agree-

ment. The alcohol extraction method was finally adopted because it gives the lower initial reduction, and because of its greater convenience for work with frozen tissue.

The reducing power of liver and muscle filtrates depends on the conditions under which the tissue is obtained. Livers obtained from normal rabbits and frozen within a minute or two after the animal was suddenly killed generally give a reduction equivalent to 100 to 125 mg. of glucose for each 100 gm. of tissue. Livers removed under amytal anesthesia and frozen within 15 to 30 seconds give much higher reducing values, with, however, about the same non-fermentable fraction (30 mg. per cent), which indicates that the difference in total reduction under these conditions is due to changes in the fermentable sugar. Muscles removed and frozen quickly after death give filtrates with variable reducing powers, 90 to 150 mg. per cent of which 40 to 50 mg. may be non-fermentable, while muscles removed under amytal anesthesia with only slight irritation, and frozen within a few seconds, give total reductions of 45 to 60 mg. per cent of which approximately half is non-fermentable.

Obviously these results show that the application of yeast fermentation to tissue extracts leads to a more exact idea of the nature of the reducing substances present.

The relatively large non-fermentable fraction obtained under some conditions during the course of this work suggests that part of this fraction is a reducing carbohydrate, non-fermentable under the conditions of these experiments. This view is supported by the older work on the subject, as well as by the more recent work of Lohmann.[33]

EXPERIMENTS WITH THE NON-GLUCOSE SUGAR OF BLOOD AND PLASMA.

By ROGER S. HUBBARD and JOHN K. DEEGAN.

(From the Laboratories of the Clifton Springs Sanitarium and Clinic, Clifton Springs, New York.)

Mixed oxalated specimens of blood and plasma obtained in the hospital for routine study were treated with yeast for short periods, precipitated with tungstic acid, and analyzed for sugar by the

[33] Lohmann, K., *Biochem. Z.*, 1926, clxxviii, 444.

Folin and Wu technique. Added glucose in concentrations of 0.2 per cent was completely destroyed, and there was no reduction given by the yeast. The average residual reducing values were approximately 0.02 per cent for blood and 0.01 for plasma expressed in terms of glucose in the original material samples. The residual reducing substances are easily destroyed by alkali and heat. A slight increase in reducing power when filtrates were treated with acid and heat was usually demonstrated. When colon bacilli were added to filtrates brought approximately to a neutral reaction and the solutions incubated 24 hours, about 0.003 per cent of reducing substance was destroyed. This was approximately evenly distributed between cells and plasma. There was in almost all cases some loss in reducing power when solutions were incubated without colon bacilli, and controls were run in all cases. Incubation continued for 2 or 3 days showed no further increase in destruction of reducing power by colon bacilli. Values somewhat higher than the average were found for the amount of material destroyed by colon bacilli in two out of three post partum cases examined. Glucose, levulose, galactose, sucrose, maltose, and lactose added to filtrates in amounts equivalent to 0.020 per cent were destroyed in 24 hours. There was no change in the amounts of these sugars if colon bacilli were not added before incubation. If filtrates to which the disaccharides had been added were treated with 0.2 N sulfuric acid and heat, hydrolysis of the added sugars could be demonstrated. Such treatment caused no change in the reducing power of the monosaccharides. Acid hydrolysis after colon treatment caused no increase in the reducing power of one specimen obtained shortly post partum when hydrolysis of the incubated control showed such an increase. In three-fourths of a series of twenty cases which received a meal rich in carbohydrate, including milk, the reducing substance was present in slightly larger amounts in a specimen drawn 2 hours after the meal than it was in a sample taken before it. The reducing substances not destroyed by colon bacilli were 3 to 4 times as concentrated in whole blood as in plasma. They were much more concentrated in four cases of severe nephritis studied than in other material. The increased reducing power seemed about equally distributed between cells and plasma. Uric acid and creatine added in amounts equivalent respectively to 10 and 30 mg. per 100 cc. of the original

material caused no change in the reducing power whether they were added to blood before fermentation or to the filtrates after treatment with yeast. Creatinine, equivalent to 10 mg. per 100 cc., added in the same way caused an increase equivalent to approximately 0.002 per cent. The creatinine content of the nephritic bloods studied was by no means high enough to account for the residual reduction values observed in them.

GLYCOLYTIC ENZYME OF THE PANCREATIC JUICE.

II. EXPERIMENTS UNDER STERILE CONDITIONS IN VITRO.

By EPHRAIM B. BOLDYREFF.

(From the Pavlov Physiological Institute of the Battle Creek Sanitarium, Battle Creek.)

In my previous publication sufficient evidence was given to show: (1) glycolytic action of pancreatic secretion *in vivo*—the decrease of the blood sugar due to secretion of pancreatic juice, and the increase of sugar content of the blood due to absence or deficiency of pancreatic secretion; (2) glycolytic action of pancreatic secretion *in vitro*—splitting of the glucose by pancreatic juice.

The pancreatic juice in my former experiments was filtered through a Berkefeld filter, which procedure was responsible for the diminished glycolysis and low production of lactic acid. The technique was therefore modified; pancreatic juice was collected under sterile conditions and the experiments were also carried out under sterile conditions as was verified by bacteriological tests. The following glucose solutions of highest purity were used: 10 per cent, 1 per cent, 0.3 per cent, and 0.2 per cent; from 5 to 10 cc. of glucose solution and from 0.5 to 1 cc. of pancreatic juice were used for each test. All specimens were incubated at 37.5°.

The intensity of glycolysis was estimated by the amount of titratable acidity as well as by direct quantitative determination of glucose (Folin-Wu).

It was found that sterile pancreatic juice possesses a strong glycolytic action. The amount of glucose split in the process was 40 to 50 per cent. Thus, the existence of glycolytic enzyme in the pancreatic juice is confirmed by the experimental evidence.

STUDIES ON THE METABOLISM OF LEVULOSE, WITH A NEW METHOD FOR ITS DETERMINATION IN BLOOD AND URINE.

By RALPH C. CORLEY.

(From the Department of Bio-Chemistry of the School of Medicine, Tulane University, New Orleans.)

A simple colorimetric method for the estimation of levulose in small amounts, in the presence of glucose, has been elaborated and applied to the analysis of blood and urine. Certain phases of the metabolism of levulose have been studied by following the changes in the concentration of levulose in the blood, and the excretion in the urine. In the normal rabbit, levulose disappeared from the blood in 90 minutes subsequent to its intravenous injection in doses of 2 gm. per kilo of body weight. The rate of removal was not strikingly affected by mild poisoning with various substances that are injurious to the liver, but more rigorous treatment with phosphorus, chloroform, and hydrazine sulfate did have an evident effect, levulose still remaining in the blood at the end of 90 minutes. The simultaneous injection of insulin with levulose did not increase the rapidity of removal of the latter from the circulation, but convulsions did not supervene even though the insulin dosage was quite large. However the previous subcutaneous or intravenous administration of insulin appeared to speed the removal of injected levulose from the blood stream, if the insulin dosage was sufficiently great. Hypoglycemic convulsions resulted in this event. The oral ingestion of levulose in quantities as large as 7 gm. per kilo of body weight caused but slight increase in the total blood sugar and the appearance of very little levulose in the circulation. Liver poisons as phosphorus, chloroform, and hydrazine sulfate, were without marked influence on the amount of levulose entering the blood stream, but the total blood sugar rose to very high levels when levulose was fed.

FASTING KETOSIS OF THE PRIMATES.

By THEODORE E. FRIEDEMANN.

(From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis.)

Experiments with seven primates, including six monkeys and one lemur, are described. The former developed a very marked ketosis which reached its maximum on about the 3rd day of fasting. A lemur (*Lemur macaco*) on the other hand excreted only traces of acetone bodies on the 3rd and 4th days of fasting. The monkeys included five European and one New World monkey, and represented a wide geographical distribution. It is pointed out that the severe fasting ketosis noted in man and in these monkeys may be due to a hereditary factor.

THE USE OF COLLOIDAL MANGANESE DIOXIDE FOR THE DETERMINATION OF LACTIC ACID.

By A. I. KENDALL and THEODORE E. FRIEDEMANN.

(From the Research Department of Bacteriology, Northwestern University Medical School, Chicago.)

Colloidal MnO_2 instead of $KMnO_4$ may be used to oxidize lactic acid to acetaldehyde by the method of Friedemann, Cotonio, and Shaffer. Overoxidation is avoided, especially when very small amounts of lactic acid (less than 0.2 mg.) are present, and the results are more uniform. The yield is the same. The yield may be increased by using 0.2 N H_3PO_4 instead of N H_2SO_4.

Colloidal MnO_2 is prepared by adding an excess of glucose to a warm alkaline solution of $KMnO_4$ (about 8 gm. of glucose and 50 gm. of $KMnO_4$ dissolved in 1 liter of 0.5 N KOH), an excess of $KMnO_4$ being finally added. The solution is then warmed on the water bath to complete the reaction, after which it is cooled. The precipitate is washed in a Buchner funnel until free from salts and suspended in water. The supernatant suspension (adjusted to about 0.1 N strength) is used for the determination.

In carrying out the lactic acid determination an excess of MnO_2 suspension may be added all at once but the reaction mixture must boil vigorously while it is being added, as otherwise the results are low. For very small amounts of glucose, as in blood, the MnO_2 suspension may be diluted.

THE INFLUENCE OF EPINEPHRINE AND INSULIN ON THE CARBOHYDRATE BALANCE OF RATS IN POSTABSORPTIVE STAGE.

By CARL F. CORI and GERTY T. CORI.

(From the State Institute for the Study of Malignant Disease, Buffalo.)

A carbohydrate balance was made on rats in postabsorptive stage by determining simultaneously sugar oxidation and disappearance of liver and body glycogen. In control rats 7.5 per cent more carbohydrate disappeared than was accounted for by oxidation; in the rats injected with epinephrine the agreement was perfect; in the rats injected with insulin 13.4 per cent more carbohydrate was oxidized than could be accounted for by the disappearance of glycogen. In the control rats 216 mg. of glycogen disappeared in 3 hours, 23 per cent being derived from liver and 77 per cent from body glycogen. In the epinephrine animals all the glycogen which disappeared (297 mg.) came from the body, there being an actual increase in liver glycogen of 26 mg. After insulin administration 43 per cent of the disappearing glycogen (329 mg.) was derived from liver and 57 per cent from body glycogen. In comparison with the control rats, the animals receiving epinephrine showed an increase in carbohydrate oxidation of 19.5 per cent and the animals receiving insulin, 97.3 per cent. The blood sugar level in the three cases was 113, 174, and 69 mg. per cent respectively. Glycosuria was not produced by the dose of epinephrine injected (0.02 mg. per 100 gm. rat). The calories of the control and insulinized animals were within 0.5 per cent the same, indicating that there occurred an equicaloric replacement of carbohydrate oxidation for fat oxidation after the insulin injection. Epinephrine caused an increase in heat production of 24 per cent, 67 per cent of the extra calories being furnished by fat and 33 per cent by carbohydrate.

A STUDY ON THE RECOVERY OF SMALL AMOUNTS OF INSULIN AND THE USE OF MICE FOR STANDARDIZATION PURPOSES.

By GEORGE W. PUCHER.

(*From the Department of Biological Chemistry, University of Buffalo Medical School, and the Department of Laboratories, Buffalo General Hospital, Buffalo.*)

A detailed study has been made on the recovery of known amounts of insulin added to insulin-free material such as egg albumin. A method has been developed whereby 0.1 to 0.2 rabbit units per 10 gm. of tissue can be detected and approximately estimated. If the amount of insulin present is between 0.5 and 2 units per 10 gm. of tissue, a consistent recovery of 30 to 40 per cent of the added insulin may be obtained.

The unknown insulin preparations are tested on 24 hour fasting white mice whose fall in blood sugar has been previously determined by intraperitoneal injection of 0.02 rabbit unit of Lilly's insulin per 20 gm. of mouse. The blood samples (30 to 80 mg.) are taken at the desired intervals from the tail vein of the animal and the sugar estimated by a modification of the Hagedorn-Jensen method. Duplicate determinations of the blood sugar of normal mice over varying periods of time up to 4 hours agree within at least 3 per cent.

THE DEAMINIZATION OF CYSTINE IN ALKALINE SOLUTIONS.

By JAMES C. ANDREWS.

(*From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia.*)

The rate of ammonia formation from solutions of pure cystine has been studied under carefully controlled conditions. The cystine solutions were heated in a bath at 100° and "aerated" with pure nitrogen at a measured rate of speed for a constant period of time. The concentration of cystine was kept constant in all cases. The rate of ammonia formation was measured in pure cystine-NaOH solutions and also after addition of various heavy metals.

From 4.0 M NaOH to about 0.2 M NaOH the percentage deaminization is *inversely* proportional to the concentration of alkali.

Under the conditions used, the percentage deaminization in 4.0 M NaOH averaged about 11 per cent. In 0.2 M NaOH it averaged 23.5 per cent. Further decrease in the concentration of alkali caused a sharp drop in percentage deaminization as the pH of the solution approached 7.

In the presence of an amount of sodium plumbite (lead acetate added) equivalent to the sulfur present, the rate of deaminization is more than tripled. 2.0 M NaOH caused 17.8 per cent deaminization without the lead and 60.0 per cent with it. Double the theoretical amount of lead raised this to 74.0 per cent; one-tenth the theoretical amount reduced it to 20.0 per cent. The effect of the lead is evidently not purely catalytic. Neither is it a catalytic effect on the part of the newly formed lead sulfide. Addition of freshly precipitated lead sulfide to the solution had no effect on ammonia production. Some years ago Van Slyke noted that the amount of ammonia derived from boiling a sample of cystine in alkali was multiplied several times if the boiling was carried on in a copper instead of a glass container. In the present experiments it was found that addition of copper salts, giving a suspension of copper hydroxide, did, in 2.0 M NaOH, increase deaminization to about 50 per cent, but that addition of pure metallic copper in similar amounts had no effect.

Addition of ferric chloride produced only a very slight rise (less than 1 per cent). On the theory that other metals which might form insoluble sulfides or slightly dissociated sulfur complexes might accelerate deaminization, experiments were carried out with addition of both zinc and arsenic salts. The results were comparable to those with iron. About 1 per cent rise in percentage deaminization was obtained. In this connection the effect of lead appears greatest and that of copper next.

Variation in the optical activity of the cystine from $[\alpha]_D = -204$ to $+8$ had no effect on this reaction, either with or without lead.

APPLICATION OF THE SPECTROPHOTOMETER TO THE DETERMINATION OF TRYPTOPHANE IN PROTEIN-FREE BLOOD EXTRACTS.

By C. A. CARY.

(From the Research Laboratories of the Bureau of Dairying, United States Department of Agriculture, Beltsville, Maryland.)

With a spectrophotometer, extinction coefficients have been determined at 560, 570, and 580 $m\mu$ for colored solutions produced from varying quantities of tryptophane by means of glyoxylic acid in the presence of H_2SO_4 and an excess of $HgSO_4$. From these data spectrophotometric determinations have been made of the tryptophane in protein-free blood extracts. The results are somewhat lower (15 to 25 per cent) than those obtained colorimetrically.

A COMPARISON OF THE DIFFUSIBLE CALCIUM OF THE SERUM AND THE CALCIUM OF THE SPINAL FLUID.

By DAVID M. GREENBERG and HERMAN E. BALLARD.

(From the Division of Biochemistry and Pharmacology, University of California Medical School, Berkeley.)

There is a fairly wide-spread belief that the spinal fluid is an ultrafiltrate in equilibrium with the blood plasma. On the basis of this, Cameron and Moorehouse[34] have proposed using the cerebral spinal calcium as a measure of the diffusible calcium of the blood. It seems desirable actually to make a direct comparison of the diffusible calcium of blood serum and calcium of the spinal fluid. If there is an equilibrium, differences are to be expected between the figures due to the use of serum rather than plasma and because of a membrane distribution effect. However, a rather constant ratio between the two calciums should be obtained if this idea is correct. Analyses were made on total calcium and diffusible calcium of human blood serum and spinal fluid calcium, the methods described in a previous communication being used.[35] No

[34] Cameron, A. T., and Moorhouse, V. H. K., *J. Biol. Chem.*, 1925, lxiii, 687.

[35] Updegraff, H., Greenberg, D. M., and Clark, G. W., *J. Biol. Chem.*, 1926–27, lxxi, 87.

agreement was found between the diffusible calcium and spinal fluid calcium, the ratio of spinal fluid calcium to diffusible calcium varying between 0.75 to over 1.5. This is against the hypothesis that the calcium of plasma and that of spinal fluid are in equilibrium with each other.

BEHAVIOR OF THE SUBSTANCE ACTIVE IN PERNICIOUS ANEMIA ON DIALYSIS OF LIVER EXTRACTS.

BY MICHAEL HEIDELBERGER, NATHAN ROSENTHAL, DAVID J. COHN, AND JOSEPH S. FRIEDMAN.

(From the Laboratories of the Mt. Sinai Hospital, New York.)

Dialyzed in parchment bags aqueous liver extract loses a portion of its activity into the outside liquid. Both fractions appear capable of causing rapid improvement in the blood picture of patients with low hemoglobin and low red cell counts, or of maintaining patients with high values at high levels.

Inside liquid, concentrated to 15 cc. to the pound of original liver: solids 31.5, 36.5 per cent; ash (as Na_2O) 4.8, 5.6 per cent.

Outside liquid, concentrated to 15 cc. to the pound of original liver: solids 46.4, 41.2 per cent; ash (as Na_2O) 16.6, 20.5 per cent.

METABOLISM OF TRYPTOPHANE.

BY RICHARD W. JACKSON.

(From the Laboratory of Physiological Chemistry, Yale University, New Haven.)

In a former paper,[36] experiments were described showing the inability of certain compounds closely related to tryptophane to replace this indispensable amino acid in a diet in which it was the limiting factor. Further similar work has been carried out to investigate the capacities of other compounds—the betaine of tryptophane (also the nitrate of the betaine), benzoyl-α-amino-β-3-indole acrylic acid (as the sodium salt), β-3-indole propionic acid, 3-indole ethylamine (as the hydrochloride), and the formaldehyde condensation product secured by treating a solution of tryptophane with formalin—to play the nutritional rôle of trypto-

[36] Jackson, R. W., *J. Biol. Chem.*, 1927, lxxiii, 523.

phane. All of these substances were found impotent in this regard. The technique employed was essentially the same as that formerly described with the variations (1) that 25 to 30 mg. of tryptophane were added to each 100 gm. of the basal diet with the purpose of bringing the rats into approximate weight balance before examining the derivatives mentioned, and (2) the cod liver oil as well as the yeast concentrate was given separate from the main diet, the more fully to insure consistent and adequate consumption of provided vitamins.

Since Asayama[37] in 1916 tested kynurenic acid from the above standpoint, various workers have investigated also the following: indole pyruvic acid, indole lactic acid, indole and alanine, indole aldehyde, and the materials aforementioned in this abstract. All of these compounds have failed to show any appreciable replacement of tryptophane under the conditions of experimentation.

THE ACTIVITY COEFFICIENTS OF CERTAIN ACID-BASE INDICATORS.

By JULIUS SENDROY, Jr., and A. BAIRD HASTINGS.

(From the Hospital of The Rockefeller Institute for Medical Research, New York.)

A study of the apparent dissociation constants of the acid-base indicators, phenol red, brom-cresol purple, and brom-cresol green, in salt solutions of varying ionic strength has been made. In the experiments with phenol red and brom-cresol purple the salts used have been NaCl, KCl, K_2SO_4, Na_2SO_4, $MgCl_2$, $MgSO_4$, and Na_2HPO_4-K_2HPO_4 mixtures. In the experiments with brom-cresol green, sodium acetate-acetic acid mixtures were substituted for the phosphates. When the logarithm of the apparent dissociation constant of any one indicator is plotted against the square root of the ionic strength, all of the results lie within a narrow sheaf of lines and conform to the general equation $pK^{1} = pK = \frac{Av^{s}}{1 + B}$.

These results can be satisfactorily interpreted as indicating that the activity coefficient of the indicator anion varies with the ionic strength in accordance with the theory of Debye and Hückel.

[37] Asayama, C., *Biochem. J.*, 1916, x, 466.

The variation in the activity coefficient of the indicator with changing salt concentration is what has been referred to in the past as the "salt error."

DOES THE FEEDING OF COD LIVER OIL PREVENT TETANY IN THYROPARATHYROIDECTOMIZED DOGS?

By ISIDOR GREENWALD and JOSEPH GROSS.

(*From the Harriman Research Laboratory, The Roosevelt Hospital, New York.*)

Of nine dogs receiving from 2.5 to 6 cc. of cod liver oil per kilo per day for from 13 to 29 days before the removal of the thyroids and parathyroids, three developed severe tetany in 2 days, two in 3 days, and one each on the 5th, 10th, and 12th days. The ninth developed slight tetany on the 3rd day, and severe tetany on the 5th day.

SOME EFFECTS OF MYRTOMEL IN THE DIABETIC DOG.

By JOSEPH A. MORRELL, J. H. VARLEY, G. W. HART, and G. SCHWOCH.

(*From the Research Laboratory, E. R. Squibb and Sons, New Brunswick.*)

These experiments were designed to observe the effects of myrtomel upon totally depancreatized dogs in which the minimum insulin requirement had been previously determined over an extended observation period.

The procedure adopted was uniform throughout all experiments. It consisted in totally depancreatizing the animal, placing it upon a liberal diet consisting of 400 gm. of chopped beef, 300 gm. of liver, and 350 gm. of white bread daily. The minimum insulin requirement to maintain the animal at reasonable blood sugar levels and with relatively sugar-free urine was determined over a considerable period of time. We found that fluctuations in the insulin requirement occurred shortly after the operation. When the dog's insulin requirement was known 1 gm. of myrtomel was given every morning in addition to the insulin. The insulin dosage was reduced about 7 days after myrtomel had been started. Sometimes a temporary hyperglycemia and glycosuria occurred

but this readjusted itself in the course of a few days. At intervals subsequent reductions were tried.

The total number of dogs in this series was eighteen. In eight dogs we succeeded in reducing the insulin requirement very markedly. In fact, in three of the eight the cut was so great that we stopped the insulin altogether, but the animals died within a few days after the insulin had been removed.

In three other dogs we brought about a reduction under the influence of myrtomel, following which we stopped myrtomel and found the insulin requirement after a few weeks increased to its premyrtomel level or even higher.

In three other dogs we were able, in the same animal, to accomplish first, insulin reduction under myrtomel, then an increase without myrtomel, and finally a second reduction under myrtomel again.

We had three failures in which we were unable to make any reduction in the insulin requirement.

One dog turned out to be a partially depancreatized animal.

THE DECOMPOSITION OF HALOGEN-CONTAINING HYDROCARBONS IN ANIMALS DURING ANESTHESIA.*

By G. H. W. LUCAS.

(From the Department of Pharmacology, University of Toronto, Toronto, Canada.)

In a previous paper were reported histological and chemical changes in the livers of rabbits following bromoform anesthesia. Chemical analyses of the livers and urine of these animals showed that about 1 per cent of the bromoform was decomposed; inorganic bromides were found in the urine and livers. The suggestion was made that the liver damage was due to the formation of the toxic carboxyl bromide as an intermediate oxidation product of the bromoform.

During the past year these investigations have been continued and evidence has been found which indicates that the livers of animals can decompose halogen derivatives of hydrocarbons *in*

* Presented before the Society for Pharmacology and Experimental Therapeutics.

vitro or *in vivo*. The distribution of the bromoform and bromides in various organs has been measured. Known quantities of a number of bromo derivative hydrocarbons have been used to produce anesthesia in rabbits and an attempt is made to relate the toxic effect with the amount of inorganic bromide found in the organs.

THE BLOOD SULFUR IN DISEASES OF THE ENERGY METABOLISM.*

By ALFRED E. KOEHLER.

(*From the Department of Medicine, Henry Ford Hospital, Detroit.*)

The demonstration that certain sulfur compounds, especially glutathione, play an important part in biological oxidation raises the question as to whether or not these sulfur compounds play a rôle in diseases of the energy metabolism. With the nephelometric method of Denis, the fasting neutral and oxidized sulfur was determined in both the red cells and plasma of 125 patients with abnormal energy metabolism and twenty-five with normal energy metabolism as determined by the basal metabolic rate.

In the normal group the plasma neutral sulfur varied from 0.5 mg. to 3.0 mg. per 100 cc. of plasma with an average of 1.4 mg. The red cells contained approximately 6 to 7 times as much neutral sulfur as was found in the plasma, ranging from 5 to 9 mg. per 100 cc. of corpuscles. The plasma sulfate varied from 1.5 to 3 mg. per 100 cc., while the sulfate contained in the corpuscles was distinctly less, ranging from a trace to 2.5 mg. per 100 cc. The average ratio of corpuscle sulfate to plasma sulfate was 1.5. The average ratio of reduced plasma sulfur to the oxidized form was 0.75.

In hypothyroidism and hyperthyroidism both the reduced and oxidized sulfur show a tendency to much greater variation with extreme values in both conditions. In several cases of acute exophthalmic goiter of short duration the neutral sulfur values were high, 14 to 16 mg. per 100 cc. of whole blood with a drop to normal after thyroidectomy. In other cases of hyperthyroidism with prolonged

* Presented before the Society for Pharmacology and Experimental Therapeutics.

undernutrition and emaciation the neutral sulfur in both plasma and corpuscles dropped below normal values.

Several cases of Addison's disease showed an elevation of the neutral sulfur fraction, 12 to 18 mg. per 100 cc. of corpuscles and 3.5 to 5 mg. per 100 cc. of plasma.

Various of the endocrine dyscrasias were studied, such as acromegaly, Frölich's syndrome, and hypogonadism, but no consistent variations in the blood sulfur were noted.

Although the diabetic shows no consistent blood sulfur variations, the intravenous injection of glucose in the sugar tolerance procedure usually raises the neutral sulfur fraction in both corpuscle and plasma, while in the non-diabetic individual the glucose injection is associated with a fall in sulfur.

The blood sulfur is apt to vary considerably in various pathological states but seems to have no definite relation to variations in the energy metabolism. Low neutral sulfur values are usually associated with states of exhaustion, undernutrition, and emaciation.

AN IMPROVED AND SIMPLIFIED METHOD FOR MAKING A STANDARD UNDENATURED TUBERCULIN OF ANY DESIRED STRENGTH AND A CHEMICAL ASSAY FOR THE SAME.*

By FLORENCE B. SEIBERT.

(*From the Otho S. A. Sprague Memorial Institute and the Department of Pathology, University of Chicago, Chicago.*)

An improved method is outlined for separating out the active principle of tuberculin. The essential features are growth of tubercle bacilli upon a liquid non-protein "synthetic" medium, and ultrafiltration of the bacteria-free Berkefeld filtrate. The advantages over all previous methods are: (1) A tuberculin of any desired potency is produced without any loss of the active principle. (2) An undenatured product is produced, as evidenced by the fact that its potency (about 0.2 mg. of protein is a minimal lethal dose for a 400 to 500 gm. tuberculous guinea pig) is roughly ten times the potency per unit of the most potent dry powder preparation previously obtained (2 mg. = minimal lethal dose).

* Presented before the Society for Experimental Pathology.

Moreover, a considerable portion of this protein will crystallize. (3) Even in the crude form the tuberculin after concentration by filtration is fairly free of concentrated irritating constituents and with very little more work it can be practically freed of all of these substances.

A chemical assay for tuberculin made upon a non-protein medium is submitted and has proved to be consistent with the biological assay (lethal test). It is based upon extensive evidence submitted in previous publications that the active principle of tuberculin is a whole protein, and that all of the water-soluble protein is active protein. Trichloroacetic acid precipitates this protein active principle quantitatively. In the filtrates which contained a small amount of active principle, as evidenced by the skin test in tuberculous guinea pigs, there was also a small amount of trichloroacetic acid precipitate formed upon standing, and conversely where there was no biological activity, there also no precipitate formed.

After concentration of four different tuberculin preparations by ultrafiltration a nearly quantitative agreement occurred in the determination of concentration made by the three different methods—amount of trichloroacetic acid precipitate, amount of nitrogen per unit volume, and the minimal lethal dose for tuberculous guinea pigs.

It was found that the potency of any tuberculin preparation increases with the age of the culture up to 3 months incubation at least. The effect of longer periods of incubation is being studied.

The tuberculin reaction, as indicated by the lethal test, is not truly specific but only quantitatively specific, since 50 to 100 times the dose for a tuberculous guinea pig produces a corresponding reaction in a normal, unsensitized animal.

A simple method for preparing and standardizing tuberculin, therefore, consists of the following steps.

1. Plant a virulent human strain of tubercle bacillus upon the Long non-protein synthetic medium and allow it to incubate 3 months.

2. Remove the bacilli completely by filtration through the Berkefeld candle and concentrate the tuberculin to any desired degree by filtering it, preserved with 0.5 per cent phenol, through alundum extraction shells previously impregnated with a 10 to

12 per cent solution of soluble guncotton in glacial acetic acid and washed free of the acid. (The rate is 15 to 20 cc. per hour through a cup 45 mm. in diameter × 127 mm.) If speed is desirable and quantitative recovery is not essential, the process may be hastened tenfold by using membranes made of an 8 per cent solution of soluble guncotton or parlodion in glacial acetic acid. Concentration by this method can be carried on to any desired degree.

3. Standardize the potency of the tuberculin by determining the amount of precipitate obtained when trichloroacetic acid is added to a standard amount of the tuberculin. Obtain the amount of protein and the minimal lethal dose by calculation (1 cc. of the precipitate corresponds to 0.0674 gm. of protein and about 0.2 mg. of this protein is a minimal lethal dose for a 400 to 500 gm. tuberculous guinea pig).

STUDIES ON THE BLOOD CALCIUM OF NORMAL AND PARATHYROIDECTOMIZED ALBINO RATS.*

By W. R. TWEEDY and S. B. CHANDLER.

(From the Department of Physiological Chemistry and the Department of Anatomy, Loyola University School of Medicine, Chicago.)

The blood plasma calcium of normal albino rats varies from 9.5 to 12.5 mg. per 100 cc. Unilateral parathyroidectomy does not appreciably change this value. After bilateral parathyroidectomy the blood plasma calcium drops very slowly, usually reaching a value of 7 to 8 mg. within 7 days. A large number of individual analyses has shown that the calcium value remains between 5 and 8 mg. for several months.

Blood plasma calcium values obtained upon the administration of standardized parathyroid extract indicate that the completely parathyroidectomized rat is 2 to 3 times more reactive than the normal rat.

According to Collip's[38] observations on dogs there is no appreciable difference in response of normal and parathyroidectomized dogs to parathyroid extract.

* Presented before the Society for Experimental Pathology.

[38] Collip, J. B., and Clark, E. P., *J. Biol. Chem.*, 1925, lxiv, 494.

ON THE INFLUENCE OF FREQUENT INTRAVENOUS ADMINISTRATION OF PHOSPHATE SOLUTIONS ON NORMAL AND NEPHRITIC DOGS.*

By ADOLPH BOLLIGER.

(*From the Laboratories of the Henry Ford Hospital, Detroit.*)

After the observation was made that the excretion of intravenously administered phosphates follows closely the functional capacity of the kidneys, the question was examined as to what the effect of the frequent intravenous administration of a rather large amount of phosphates would be, particularly in an animal with kidney impairment.

420 to 470 mg. of phosphorus in the form of sodium phosphate buffer solutions, pH varying from 7.1 to 8.0, were administered at least once weekly (usually twice and sometimes three times) to normal and to nephritic dogs with different degrees of kidney impairment, for periods ranging from 1 to 15 months. The nephritis was of the chronic interstitial type and was produced by x-ray. At frequent intervals the 2 hour elimination of phosphates in the fasting animal was determined quantitatively and at the same time blood chemistry was studied and the excretory function evaluated with the phenolsulfonephthalein test.

A. Normal Animals.—Five dogs were studied for periods varying from 1 to 6 months. An apparently normal animal was studied for 1 year. In this case phosphates of the quantity and range of pH mentioned above were given 72 times. Also on several occasions acid phosphates were given of pH 4.6. So far none of these animals has shown any functional impairment excepting a moderate albuminuria.

B. Dogs with Slight Slowly Progressive Kidney Impairment.—To a group of six dogs, which had had x-ray treatment through the abdominal wall and which showed definite but rather slight functional deficiency (blood urea nitrogen 20 to 35 mg., phenolsulfonephthalein test 45 to 60 per cent, low urine specific gravity, albuminuria, and hematuria), phosphates were administered for 6 to 14 months. Two of these animals are still living. Today their nephritis is the same or even less in degree than it was before

* Presented before the Society for Experimental Pathology.

phosphate administration. The other four animals in this group, observed for a shorter time, showed similar results.

C. Dogs with Rapidly Progressing or Far Advanced Nephritis.—Phosphate buffers were administered to a series of six such animals with somewhat varying results. These results varied with the different conditions and the final complications which occurred in these animals. In one animal, which is still alive, phosphates were started after a rapid initial downhill course. Blood urea nitrogen was then from 60 to 85 mg., phenolsulfonephthalein test around 40 per cent. From previous experience on about thirty animals it was estimated that the life of this animal would not last more than 2 months. After 7 months of phosphate administration the animal is still in very good condition, having the appearance of a strong normal dog. In spite of severe nitrogen retention and marked acidosis already lasting for 5 months, it never showed any signs of uremic toxemia. The phosphates administered in this case were of a pH from 7.6 to 8.0. Another similar animal received phosphates of pH 7.2. This dog remained in very good condition notwithstanding low function (phenolsulponephthalein test 20 to 30 per cent) up to the last few days. At autopsy this animal showed a large stone in the renal pelvis. Good physical conditions were also observed in other animals treated for a shorter period. These animals died of infections of various kinds, complicating the far advanced renal disease. The course of the renal disease in injected animals was compared with a large series of dogs with similar deficiency.

In reviewing these experimental findings it was concluded that the phosphates injected intravenously may serve as a fair experimental renal function test. They do not produce any progressive renal damage in normal animals. In the animal with slowly progressive chronic renal lesions the progress of the disease was definitely arrested, while in animals with advanced renal impairment administration of phosphates was an important factor in maintaining fair nutrition, avoiding uremic toxemia, and prolonging life.

IMMUNOLOGICAL RELATIONSHIPS OF THE TYPHOID BACILLUS.*

By MICHAEL HEIDELBERGER, GREGORY SHWARTZMAN, AND DAVID J. COHN.

(From the Laboratories of the Mt. Sinai Hospital, New York.)

Rabbits immunized with the cell-free whole filtrate of a strain of typhoid bacillus gave antisera which agglutinated as strongly as those prepared from whole cells, but which failed to precipitate purified carbohydrate fractions of the bacillus. Thus, as in the case of pneumococci, streptococci, and Friedländer bacilli, immunization with intact cells appears necessary for the production of antibodies which react with the carbohydrate fraction. Attempts failed to separate the acetic acid-precipitable protein of the typhoid bacillus into fractions of different isoelectric points. The protein acts antigenically and yields strongly agglutinating sera which precipitate the protein antigen but not the carbohydrate fraction. The occurrence of large or small flocks in the agglutination and precipitin tests in the various sera appears to depend on the proportions of antigen (or haptene) and antibody present rather than on the chemical differences between the two antigenic factors included in this study.

THE RATE OF ELIMINATION OF UREA AS A MEASURE OF KIDNEY FUNCTION.*

By ROBERT C. LEWIS AND INEZ H. MATTISON.

(From the University of Colorado, Denver.)

Rabbits with nephritis produced by relatively small injections of arsenic, uranium, or tartrate showed a significant delay in the elimination of urea as compared to that of normal animals, even when the urea and total non-protein nitrogen of the blood and the phenolsulphonephthalein excretion were normal.

Similar findings were obtained in man. As compared to a maximum excretion in the 1st hour after ingestion of 5 gm. of urea in thirty-eight normal men and women, a number of individuals having albumin and casts, or albumin alone, in the urine showed

* Presented before the Society for Experimental Pathology.

a maximum urea excretion in the 3rd to 4th hour, although blood chemistry and phenolsulphonephthalein excretion remained normal. In orthostatic albuminuria there was no such delay.

A slowing of the rate of elimination of urea followed the removal of one kidney from each of six rabbits. This delayed excretion continued even 4 months after operation, although at no time did the animals show other evidence of disturbed kidney function. A similar finding was obtained with a man who had had a unilateral nephrectomy following an accident.

Our findings show that the rate of elimination of urea is a much more sensitive index of kidney function than the tests ordinarily employed.

SULFUR METABOLISM AND PARTITION OF SULFUR IN THE URINE OF FASTING DOGS.[39]

By SERGIUS MORGULIS.

THE EFFECT OF INSULIN ON PROTEIN METABOLISM.[40]

By VEON CARTER KIECH and JAMES MURRAY LUCK.

A SIMPLE RESPIRATION APPARATUS FOR DETERMINATION OF OXYGEN AND CARBON DIOXIDE IN INDIRECT CALORIMETRY.[41]

By J. F. McCLENDON, HILDING C. ANDERSON, F. R. STEGGERDA, CLAIRE CONKLIN, and MILDRED WHITAKER.

HEAT INACTIVATION OF CATALASE AT DIFFERENT HYDROGEN ION CONCENTRATIONS.[42]

By SERGIUS MORGULIS and M. BEBER.

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---

[39] Morgulis, S., *J. Biol. Chem.*, 1928, lxxvii, 627.

[40] Kiech, V. C., and Luck, J. M., *J. Biol. Chem.*, 1928, lxxviii (in press, July).

[41] McClendon, J. F., Anderson, H. C., Steggerda, F. R., Conklin, C., and Whitaker, M., *J. Biol. Chem.*, 1928, lxxvii, 413.

[42] Morgulis, S., and Beber, M., *J. Biol. Chem.*, 1928, lxxvii, 115.

[43] Chanutin, A., and Beard, H. H., *J. Biol. Chem.*, 1928, lxxviii, 167.

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---

[44] Shohl, A. T., Bennett, H. B., and Weed, K. L., *J. Biol. Chem.*, 1928, lxxviii, 181.

[45] Calvery, H. O., *J. Biol. Chem.*, 1928, lxxvii, 489.

[46] Bischoff, F., Blatherwick, N. R., and Sahyun, M., *J. Biol. Chem.*, 1928, lxxvii, 467.

[47] Wintersteiner, O., du Vigneaud, V., and Jensen, H., *J. Pharmacol. and Exp. Therap.*, 1928, xxxii, 397.

[48] du Vigneaud, V., Jensen, H., and Wintersteiner, O., *J. Pharmacol. and Exp. Therap.*, 1928, xxxii, 367.

[49] Sherman, H. C., Quinn, E. J., Day, P. L., and Miller, E. H., *J. Biol. Chem.*, 1928, lxxviii, (in press, July).

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---

[60] Williams, R. R., and Waterman, R. E., *J. Biol. Chem.*, 1928, lxxviii, (in press, July).

[61] Andrews, J. C., *J. Physic. Chem.*, 1928, in press.

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[52] Somogyi, M., *J. Biol. Chem.*, 1928, lxxviii, 117.